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**Preparation of a self-contained NADH co-
factor recycling particle system**

Thesis presented for the degree of

Master of Science

In the Department of Molecular and Cell Biology

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by

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Abstract

Oxidoreductases are interesting enzymes with potential applications in a number of different industries such as the textile, food and feed, chemical and biomedical industries. Oxidoreductases require the use of co-factors. These small molecules are relatively expensive and are required in stoichiometric amounts for their enzymatic reaction; this negatively impacts the economic viability of their potential applications. Several methods have been developed to counteract this problem, the most preferred of which is the enzymatic co-factor recycling method. A few methods for the co-immobilisation of enzymes and co-factors have been developed. These systems are of interest as they offer the advantages of recycling the enzymes together with the co-factor, thereby enabling re-use. The immobilisation of enzymes also provides a platform for improving their stability, activity, specificity and selectivity. Since glucose dehydrogenase (GDH) and NADH oxidase, are industrially relevant co-factor recycling enzymes for NAD(P)H and NAD⁺ respectively, characterisation of their immobilisation is of interest.

The current work describes the use of the proprietary particle technology, termed ReSyn™, for the construction of a self-contained co-factor recycling system. The research included the optimisation of immobilisation for the individual enzymes, followed by the co-immobilisation with subsequent co-factor entrapment. The immobilised enzymes displayed improved thermal and pH stability compared to the non-immobilised enzymes. Immobilised GDH also displayed increased activity over the acidic range when compared to free GDH. The system was shown to be capable of recycling NADH/NAD⁺ up to at least 142 times with a specific activity of 10.18 U.mg⁻¹. The system was recovered and recycled with a 77% activity efficiency indicating recovery of the system and reusability.

Preparation of a functional self-contained co-factor recycling system was demonstrated consisting of the biological components NADH oxidase and glucose dehydrogenase, immobilised on a polyethylenimine support with entrapped co-factor. This serves as proof-of-principle for the construction of derivative systems

that could be used for the development of applications such as efficient biosynthesis, novel biosensors, diagnostic and therapeutic systems.

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List of Abbreviations

ADH	Alcohol dehydrogenase
BSA	Bovine serum albumin
CD	Circular dichroism
CLE	Cross-linked dissolved enzymes
CLEA	Cross-linked enzyme aggregates
CLEC	Cross-linked enzyme crystals
ELISA	Enzyme-linked immunosorbent assay
FDH	Formate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
GDH	Glucose dehydrogenase
GluDH	Glutamate dehydrogenase
LDH	Lactate dehydrogenase
NAD ⁺	Nicotinamide adenine dinucleotide free acid
NADH	Nicotinamide adenine dinucleotide hydrate
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrate
NOD	NADH oxidase
NP-4	Nonoxynol-4
PDH	Phenylalanine dehydrogenase
PEG	Polyethyleneglycol

PEI	Polyethyleneimine
PSD	Particle size distribution
ROS	Reactive oxygen species
TEA	Triethylamine
TTN	Total turnover number
PEG-NADH	Polyethyleneglycol linked nicotinamide adenine dinucleotide hydrate
PEG-NAD ⁺	Polyethyleneglycol linked nicotinamide adenine dinucleotide

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Chapter 1

General Introduction

1.1 Enzymes

Enzymes are biological catalysts that are responsible for the multitude of highly specific chemical reactions that occur in biological organisms (Cao, 2005a). The highly specific nature of enzymes as catalysts has resulted in various applications in the food and feed, material, chemical, textile and in pharmaceutical industries (Costa *et al.*, 2004). The superior efficiency, highly specific and comparatively clean technology (reduced byproducts) has resulted in the use of these biological catalysts for the production of fine chemicals; this application is known as biocatalysis (López-Gallego & Schmidt-Dannert, 2010). One group of enzymes which has received much attention are the oxidoreductases, these represent about one quarter of all known enzymes (Liu & Wang, 2007). This group is comprised of dehydrogenases (E.C. 1.1- to 1.5-), oxidases (E.C. 1.6-) and reductases (E.C. 1.7-) (Hummel & Kula, 1989; Hummel, 1999). Oxidoreductases have found application in the analytical and clinical industries for the development of biosensors; in food beverages and pharmaceutical industries for the synthesis of chiral precursors such as alcohols, aldehydes and acids; for bioremediation such as the degradation of organic pollutants; and in the textile and polymer industries for the preparation and degradation of polymers, particularly biodegradable and biocompatible polymers (Sheldon & Stephen, 1983; Hummel & Kula, 1989; Hummel, 1999; Liu & Wang, 2007). Although an extensive range of applications for enzymes are being developed, they are currently not without limitation. The applications of enzymes are limited by their relative instability, including short-half-lives, instability at high temperatures and in highly acidic or alkaline conditions, and further environmental parameters including their use in organic solvents (Mateo *et al.*, 2007).

1.2 Methods for improving enzyme activity and stability

1.2.1 Protein engineering

Protein engineering, which involves both components of rational design and directed evolution, is a tool which has been used to optimise enzymes for improved catalytic activity, enhanced physical stability, substrate specificity, and stereoselectivity (Turner, 2003). Rational design is a method of protein engineering that requires in depth knowledge of the enzyme structure, the relationship between sequence, structure and function and/or reaction mechanism. From this information, molecular modelling can be used to predict how to improve the enzyme, usually using mutagenesis or amino acid substitutions (Bornscheuer & Pohl, 2001). Directed evolution, an alternative approach to protein engineering, combines the generation of random genetic libraries (e.g. using error-prone polymerase chain reaction) and strategies for the selection of variant enzymes that possess the specific characteristics of interest (Turner, 2003). This approach allows for fortuitous discoveries and can be used to identify important amino acids that can subsequently be used for rational design. The limitations of directed evolution are finding a suitable screening and selection method for variants with desired attributes; and the practicality of evaluating large libraries, e.g. conducting laborious activity assays (Dalby, 2007).

An example of where protein engineering has been used to improve the properties of an enzyme is the case of alcohol dehydrogenase (ADH) from the thermophile *Pyrococcus furiosus*. This enzyme was engineered for improved activity at low temperatures for the production of enantiopure (2S, 5S)-hexanediol using a single round of error-prone polymerase chain reaction. The greatest improvement was a 10-fold increase in activity at 30 °C compared to the activity of the wild-type enzyme (Machielsen *et al.*, 2008).

1.2.2 Chemical modification

Chemical modification is one of the oldest tools used in improving enzyme stability, dating back to the 1960's (Polizzi *et al.*, 2007). Initial studies began with the covalent modification of enzymes and crosslinking with chemicals such as glutaraldehyde (Polizzi *et al.*, 2007). The modifications have subsequently been extended to the use of various polymers (Polizzi *et al.*, 2007). Phenylalanine dehydrogenase (EC 1.4.1.20) from *Bacillus badius* was chemically modified by glycosidation using functionalised β -cyclodextrin derivatives. The activity of the octameric enzyme was retained with a 10 °C increase in the optimum temperature, that is, from an optimum of 40 to 50 °C (Villalonga *et al.*, 2006). When a thermostable glucose dehydrogenase (GDH) from a moderate thermophilic bacterium, SM4, was chemically modified by cross-linking with glutaraldehyde an improved retention in activity was observed. More than 80% of activity in the cross-linked enzyme during elevated temperature incubation for 30 min at 65 °C was observed as compared to the 10% retained by native GDH. The half-life of the free enzyme at this temperature was 2.5 min while that of the cross-linked enzyme was 72 min, a 29-fold improvement (Yamazaki *et al.*, 1999).

1.2.3 Immobilisation

Enzyme immobilisation has been shown to lead to improved properties such as stability, activity, specificity and selectivity; and further facilitates recovery of enzymes for re-use (Cao *et al.*, 2003; Cao, 2005a, b; Liu & Wang, 2007; Mateo *et al.*, 2007; Polizzi *et al.*, 2007). The applications of immobilised enzymes include bioremediation, bioanalytical, biomedical applications, pharmaceuticals, biocatalysis, polymerase chain reaction, protein digestion in proteomic analysis and biofuel cells (Liang *et al.*, 2000; Kim *et al.*, 2006). Enzyme immobilisation can be divided into two distinct categories: self and solid support immobilisation.

1.2.3.1 Self immobilisation

Self-immobilisation (carrier-free immobilisation) can be achieved by directly crosslinking enzymes into crystalline, aggregated and spherical enzyme particles. Self-immobilisation technologies that have been developed include cross-linked dissolved enzymes (CLEs), cross-linked enzyme crystals (CLECs), cross-linked enzyme aggregates (CLEAs) (Cao *et al.*, 2003) and more recently Spherezyme technology (Brady *et al.*, 2008). The CLE method suffered several setbacks which included low retention of activity, poor reproducibility, low mechanical stability and difficulty in handling due to their gelatinous nature; to overcome these drawbacks an alternative method was developed known as CLECs (Schoevaart *et al.*, 2004). Enzymes such as, ribonuclease A, carboxypeptidase B, alcohol dehydrogenase and certain lipases, displayed broad pH stability, thermostability, resistance to proteolysis and enhanced stability in organic solvents when they were immobilised using the CLEC technology (Cao *et al.*, 2000; Cao *et al.*, 2003; Sheldon, 2007; Roessl, *et al.*, 2010). Despite the improvement in stability, which was attributed to the high mechanical stability resulting from the crystallisation of the enzyme (Brady & Jordaan, 2009; Roessl *et al.*, 2010), an inherent disadvantage of CLEC's is the requirement for highly purified enzymes followed by a laborious crystallisation procedure of the enzymes has hampered further developments and applications of this technology (Schoevaart *et al.*, 2004; Brady & Jordaan, 2009; Roessl *et al.*, 2010).

In an attempt to overcome the limitations of CLEC's, Cao *et al.* (2000) developed CLEA technology. Enzyme products from this technology have been commercialised (<http://www.cleatechnologies.com>). The preparation of CLEA's involves the precipitation of enzymes to form aggregates with one or a combination of several enzymes (Sheldon *et al.*, 2005) using salts, solvents, non-ionic polymers or acids (Cao *et al.*, 2000; Schoevaart *et al.*, 2004). The aggregates are subsequently cross-linked to achieve a stable, all-protein precipitate. Crosslinking occurs by using bi-functional cross-linkers such as glutaraldehyde or polymers such as glutaraldehyde-ethylene diamine or dextran aldehyde (Brady & Jordaan, 2009; Roessl *et al.*, 2010). CLEA's of different enzymes differ in their catalytic behaviour which has been associated with the

crosslinking agent used (Cao *et al.*, 2003; Roessl *et al.*, 2010) CLEA's were prepared from penicillin acylase, alcohol dehydrogenase (ADH) and two nitrilases were immobilised using this method in the presence of glutaraldehyde; the activity of ADH and the nitrilases was totally lost, while a 48% activity retention was observed for penicillin acylase. When poly-aldehyde was used for crosslinking ADH retained 7%-10% activity, the nitrilases 50%-60% activity and penicillin acylase's retention in activity increased to 90% (Mateo *et al.*, 2004). Furthermore, it has been reported that the precipitants used during aggregation had an effect in the catalytic behaviour of the subsequent CLEA (Sheldon *et al.*, 2005). This variation in enzyme activity was attributed to alternate enzyme structural conformations with interdependence on the duration of the precipitation step (Sheldon *et al.*, 2005; Brady & Jordaan, 2009).

The aggregation and crosslinking of more than one enzyme termed Combi-CLEA enables the possibility of multi-step biocatalytic transformations (Roessl *et al.*, 2010). Mateo *et al.* (2006) co-immobilised oxy-nitrilase from *Manihot esculenta* and a non-selective recombinant nitrilase from *Pseudomonas fluorescens* EBC 191 and the enzymes retained activity with the product (S)-mandelic acid formed in high yields from benzaldehyde.

1.2.3.2 Solid support immobilisation

Solid support immobilisation (carrier-based immobilisation) involves either encapsulation, entrapment or the physical binding of enzymes onto a solid support (Cao *et al.*, 2003). Entrapment and encapsulation protect enzymes by minimising contact with the external, often harsh, environment. However, a common drawback with these methods of immobilisation is mass transfer limitations (Brady & Jordaan, 2009). This feature limits their application for catalysis of large substrates and/or reactions which produce relatively large products. The physical binding of enzymes can be achieved by adsorption, ionic and covalent binding to improve enzyme stability and catalytic activity (Cao, 2005b; Brady *et al.*, 2009). The choice of method is selected to suit the intended application (Cao, 2005a). Adsorption and ionic binding often result in enzyme leaching off the support since

the bonds are generally weak and reversible in certain environmental conditions (Brady & Jordaan, 2009). The advantage of using this method of immobilisation is that the weak interactions allow for the re-use of the often expensive support material (Brady & Jordaan, 2009; Cao *et al.*, 2003). On the other hand, covalent binding alleviates this problem but does not allow re-use of the support (Cao *et al.*, 2003). Covalent binding occurs mainly through ϵ -amino groups of lysine residues found in proteins (Křenkova & Foret, 2004). To achieve covalent binding, supports usually contain aldehyde (Yong *et al.*, 2010), epoxide or glyoxyl functionality (Mateo *et al.*, 2007) or may be activated with chemicals such as glutaraldehyde (Filho *et al.*, 2008) or cyanogen bromide (Schnapp & Shalitin, 1976). The epoxy activated supports are capable of reacting with a variety of different amino acid functional residues on proteins including amino, thiol, hydroxyl, imidazole and carboxylic groups (Filho *et al.*, 2008). The proteins become immobilised on areas where there is a high density of these reactive residues resulting in multi-point attachment which leads to enzyme stabilisation (Mateo *et al.*, 2007; Filho *et al.*, 2008). Many industrially interesting enzymes are multimeric including dehydrogenases, oxidases, catalases, aldolases and galactosidases. The drawback of their multimeric nature is that they are inactivated through the incorrect assembly of their subunits (Mateo *et al.*, 2007; Filho *et al.*, 2008). When exposed to harsh conditions, multi-point attachment is considered essential to prevent distortion and subunit dissociation (Mateo *et al.*, 2007; Filho *et al.*, 2008; Fernández-Lafuente, 2009).

Multipoint attachment was shown in the immobilisation of formate dehydrogenase (FDH) on glyoxyl agarose, resulting in improved stability of the dimeric enzyme (Bolivar *et al.* 2006a). At acidic pH, where subunit dissociation is the first step in enzyme inactivation, the glyoxyl-agarose immobilised FDH displayed a 10°C increase in the optimal temperature at pH 4.5. Furthermore, at 65 °C (pH 4.5) the immobilised FDH retained 65% of its activity whilst the free enzyme remained inactive under these conditions (Bolivar *et al.*, 2006a). Filho *et al.*, 2008 immobilised hexameric α -galactosidase on epoxy, glyoxyl and glutaraldehyde activated supports, all preparations exhibited more stability as compared to their native soluble enzyme. The greatest improvement in stability was observed for glutaraldehyde activated supports with an increased enzyme half-life of over 1000-

fold at pH 7.0 and 75 °C. This included an increase in the optimum temperature of 5 °C, from 65 °C to 70 °C. The enhanced activity of enzymes at elevated temperatures is desirable for processes such as waste water treatment, bleaching during pulp and paper processing, chiral synthesis of compounds, production of high glucose syrup, and the production of detergents (Bruins *et al.*, 2001)

In addition to the requirement of a high density of reactive binding groups, a large surface area is also required to allow for high enzyme loading. Low enzyme loading results in low volumetric specific activities (Adlercreutz, 1997). A large surface area also reduces the occurrence of steric hindrance and mass transfer limitations which may reduce the specific activity of the immobilised enzyme (Adlercreutz, 1997). For example, a support with a large pore size can permit enzyme penetration during immobilisation, and allow movement during catalytic activity and the diffusion of substrates and products during the reaction (Adlercreutz, 1997). It is evident that the application conditions should be considered to identify a suitable immobilisation method and/or support since they may fulfil some, but not all, of the desired characteristics (Mateo *et al.*, 2007).

The characteristics of supports are not the only determining factors in successfully immobilising enzymes; immobilisation conditions also play an important role. These conditions include the reaction time, pH, temperature, and types of buffers (Mateo *et al.*, 2007). Optimal immobilisation conditions vary according to the type of support and the enzyme. Obtaining the right combination of conditions is considered a matter of trial and error (Cao, 2005a; Mateo *et al.*, 2007; Illanes *et al.*, 2010). The catalytic duration of the reaction is essential to allow the reaction between the enzyme and the support and further allows the correct alignment of groups for the immobilisation reaction to take place (Mateo *et al.*, 2007). Moderately high temperature have been said to favour immobilisation and has been ascribed to the increased vibration of the constituents, leading to increased interactions between the support and enzymes (Mateo *et al.*, 2007). This method of immobilisation may not be favourable for all enzymes as they may be deactivated at elevated temperatures (Bahar & Celebi, 1999). Many immobilisation protocols can be performed at neutral pH, but immobilisation via the lysine residues requires alkaline conditions due to the increased reactivity for nucleophilic attack (Mateo *et al.*, 2007). Buffer selection is important as it can

interfere or take part in the immobilisation reaction (Mateo *et al.*, 2007). Buffers such as Tris or ethanolamine have primary amine groups which may compete with the lysine groups of the protein for epoxide support immobilisation (Mateo *et al.*, 2007). From this information it is evident that the right combination of support characteristics and conditions for immobilisation can improve enzyme stability for meeting the expectations of intended applications.

1.2.3.3 Combination of solid support and self immobilisation

The stabilisation of enzymes through immobilisation can further be performed by a combination of the aforementioned methods. For example, when lipase B from *Candida antarctica* was chemically modified using the bifunctional reagent ethylene glycol bis(succinimidyl succinate) followed by immobilisation on nitrile-modified mesoporous silica. This method of immobilisation resulted in a 60-fold increase in the stability at 70 °C when compared to the soluble enzyme (Forde *et al.*, 2010).

1.3 Oxidoreductases

Enzymes falling in the class of oxidoreductases are ubiquitous in nature and occur in microbes, plants and animals (Xu, 2005). These enzymes have found favour in the synthesis of amino acids, such as L-leucine, L-alanine, L-phenylalanine using the enzymes L-leucine, L-alanine, L-phenylalanine dehydrogenase respectively; and hydroxyl acids such as 3-(3,4- dihydroxyphenyl)lactic acid using hydroxyl isocaproate dehydrogenase (Hummel, 1999). The redox reactions catalysed are facilitated by the biologically derived chemical species termed co-factors (Liu & Wang, 2007; Kohlmann *et al.*, 2008). Unlike enzymes, co-factors are non-catalytic and therefore, are required in stoichiometric quantities (Liu & Wang, 2007). Applications of these co-factor dependent enzymes is complicated by the expense of the co-factor in relation to the desired reaction products, and can further complicate down-stream processing (contaminant) (Liu & Wang, 2007; Kohlmann *et al.*, 2008). The co-factors may further be lost during the reaction and thereby result in non-continuous, non-viable applications (Liu & Wang, 2007). These

limitations of oxidoreductases has necessitated the development of mechanisms for the re-use, or recycling of co-factors.

1.4 Methods of co-factor recycling

Several methods of co-factor recycling have been explored and evaluated to enable the potential applications of oxidoreductases (Kohlamnn *et al.*, 2008). These methods include the use of whole cells, chemical, electrochemical, photochemical and enzymatic systems (Wichmann & Vasic-Rački, 2005; Liu & Wang, 2007; Kohlamnn *et al.*, 2008). The advantages and disadvantages of these methods are presented in Table 1.1.

Whole cell systems are preferred due to their ease of handling, non-toxicity and broad substrate tolerance (Kratzer *et al.*, 2008). However, their applicability is hampered due to low optical purities resulting from the presence of enzymes with overlapping substrate specificities but different enantio and stereoselectivity (Kratzer *et al.*, 2008). Large quantities of living cells are required for productivity which is not ideal as cellular metabolites may complicate down-stream processing, and due to the highly diluted media (required to ensure cell viability) this may further affect product recovery from the reaction medium (Wichmann & Vicki-Rački, 2005). With respect to the chemical methods of co-factor recycling, the use of dihydrogen (H_2) appears to be common and is regarded as relatively inexpensive (Abril & Whitesides, 1982; Wichmann & Vicki-Rački, 2005). It is further favoured because it does not yield any by-products (Abril & Whitesides, 1982; Kratzer *et al.*, 2008). To work efficiently this system must generate a reduced co-factor with very high chemical yield and 1,4-regioselectivity in conditions which are compatible with requirements for enzyme activity (Wichmann & Vicki-Rački, 2005). The reported drawbacks of chemical co-factor recycling methods are the cumbersome reaction conditions, expensive and usually toxic reagents, and/or unwanted side reactions (Wichmann & Vicki-Rački, 2005).

The photochemical method of co-factor recycling can be done by means of reductive or oxidative regeneration (Willner & Mandler, 1989). An example of

reductive recycling is the use of a photogenerated N, N' dimethyl-4,4'-bipyridium radical cation which acts as an electron carrier to the enzymes involved in the recycling reaction this has been demonstrated with lipoamide dehydrogenase and ferredoxin reductase (Steckhan, 1994). In oxidative systems excited photosensitiser dyes such as acridine dyes are reductively quenched by NAD(P)H to generate the oxidised form of the cofactor (Willner & Mandler, 1989). The major disadvantages of this method are the low regioselectivity (Willner & Mandler, 1989) leading to co-factor inactivation by means of dimer cofactors being formed (Willner & Mandler, 1989; Julliard *et al.*, 1986; Berenguer-Murcia & Fernandez-Lafuente, 2010), occurrence of side reactions and a low total turnover number (Chenault & Whitesides 1987; Wichmann & Vicki-Rački, 2005).

The most preferred methods are the electrochemical and enzymatic methods. The electrochemical recycling method may be accomplished by directly recycling the co-factor from the electrode surface or facilitated by an enzyme-catalysed reaction (Figure 1.1a-b respectively) (Kohlmann *et al.*, 2008). The enzyme-catalysed electrochemical co-factor recycling method is established for analytical purposes, particularly biosensor applications, but in most cases it lacks in long term stability for synthetic applications due to electrode fouling by adsorption of the substances from the solution and large over-potentials (Wichmann & Vicki-Rački, 2005; Kohlmann *et al.*, 2008). These limitations can be overcome by using chemically modified electrodes that contain mediators which substantially lower the high voltage for NADH oxidation (Wichmann & Vicki-Rački, 2005).

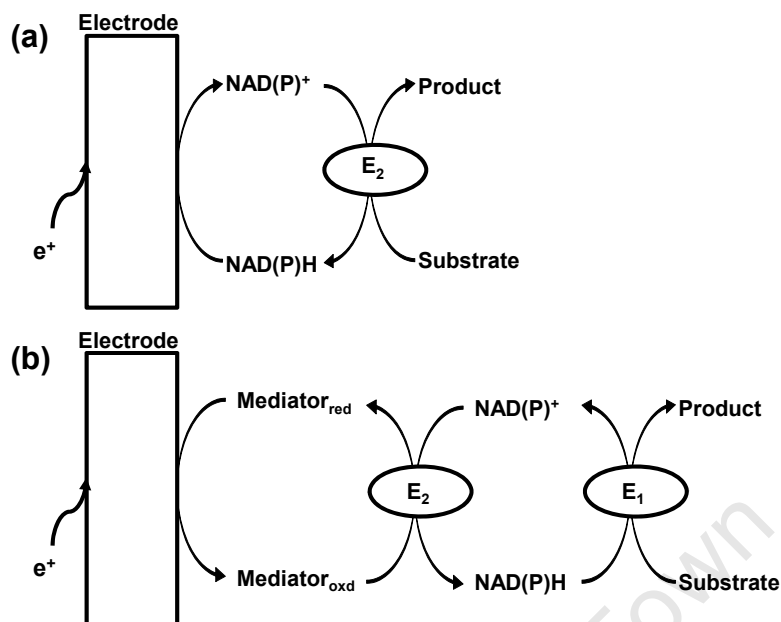


Figure 1.1: Enzyme-coupled electrochemical co-factor recycling reactions. (a) The co-factor is recycled by undergoing redox reactions directly on the electrode. (b) the co-factor is recycled through an enzyme-catalysed reaction; the redox reactions on the electrode regenerate the second substrate, the mediator. E_1 and E_2 refer to different enzymes (Reproduced from Liu & Wang, 2007).

The enzymatic method has been the most preferred since it has desirable catalytic specificity, selectivity and efficiency with minimal or no side reactions involved (Zhao & van der Donk, 2003; Wichmann & Vicki-Rački, 2005; Liu & Wang, 2007; Kohlmann *et al.*, 2008). Furthermore, they can be engineered for improved properties with respect to co-factor recycling (Section 1.2). The major hindrance to the use of enzymes is their relative expense (Mateo *et al.*, 2007; Liu & Wang, 2007). The enzymatic co-factor recycling method can be divided into two sub-groups: the substrate-coupled and enzyme-coupled reaction systems (Fig. 1.2). The substrate-coupled method utilises one enzyme which is equally capable of using the reduced and oxidised form of the co-factor using different substrates to drive the forward and recycling reaction (Wichmann & Vasic-Rački, 2005). Findrik *et al.*, 2005 applied this method using alcohol dehydrogenase (ADH) from *Thermoanaerobacter* sp. to convert acetophenone to (S)-1-phenylethanol with the concomitant oxidation of NADPH to NADP^+ . A second enzyme substrate, 2-propanol was used for recycling the co-factor back to reduced form (NADPH). The limitation of using the same enzyme is associated with reaction equilibrium as it

requires high concentrations of substrate to drive the two different reactions within the same reaction medium (Liu & Wang, 2007).

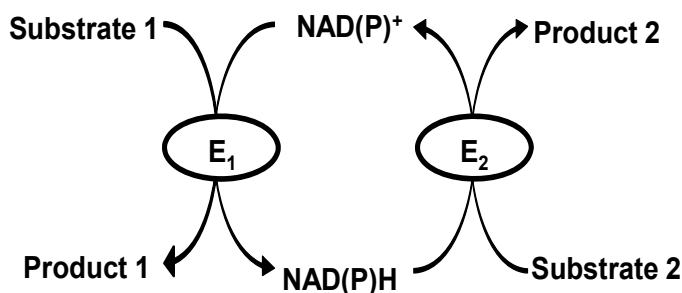


Figure 1.2: Enzymatic co-factor recycling reactions (the enzymes are the same in substrate-coupled co-factor recycling, $E_1=E_2$; the two enzymes are different in enzyme-coupled co-factor recycling, E_1 and E_2). (Adapted from Liu & Wang, 2007).

The enzyme-coupled method requires the use of two different enzymes; this becomes highly beneficial if the second enzyme also results in a valuable reaction product (Liu & Wang, 2007). This method has been adopted for the majority of co-factor recycling processes since it simplifies process parameters such as thermodynamic equilibrium. It is further preferred for therapeutic and other clinical applications of co-factor recycling (Liu & Wang, 2007). A number of enzymes combinations and methods have been applied in the recycling of NAD(P)H, these include those shown in Table 1.2.

Most enzyme based co-factor recycling methods involve immobilisation on membranes which results in mass transfer limitations (Table 1.2a) (Liu *et al.*, 2009). Soluble enzymes are also used in various systems but this is not ideal for expensive enzymes as the process may not be economically viable (Table 1.2b-c) (Mateo *et al.*, 2007). A recent method explored by Liu *et al.*, 2009 (Table 1.2d) involved the immobilisation of the enzymes, GluDH and LDH, and co-factor on separate nanoparticles in constructing an immobilised co-factor recycling system. For the reaction to occur, it relied on the mobility of the particles to generate particle-particle interactions that allowed the surface-bound co-factor to coordinate with the enzymes (Liu *et al.*, 2009). The surface based immobilisation may results

in exposure to harsh reaction conditions due the lack of a micro-environment, limiting enzyme stability which is likely the major limitation to this system (Mateo *et al.*, 2007).

Table 1.1: Advantages and disadvantages of various co-factor recycling methods (Adapted from Wichmann & Vasic-Rački, 2005).

Method	Advantages	Disadvantages
Biological (whole-cells and enzymatic)	<ul style="list-style-type: none"> - Inexpensive, self-assembled enzyme systems - Inexpensive regenerating reagents (nutrients) - High selectivity 	<ul style="list-style-type: none"> - Low reactor volume productivity from slow recycling - Contamination with biological products results in complicated product isolation - Limited stability - Reduced enantiomeric purity of product due to the possibility of various undesired reaction products - Difficulty in controlling relative activities of enzymes - Possible incompatibility with some chemical or biochemical components (cells must remain viable)
Enzymatic: Substrate and enzyme coupled co-factor recycling	<ul style="list-style-type: none"> - High selectivity - Compatibility with enzyme-catalysed synthesis - High reaction rates - Easy monitoring of reaction progress - Defined products from recycling 	<ul style="list-style-type: none"> - Enzyme cost and instability - Additional reagents can complicate desired product isolation in some cases - Low reactor volume productivities for some systems

Method	Advantages	Disadvantages
Electrochemical	<ul style="list-style-type: none"> - Lower cost - No stoichiometric regenerating reagent required - Readily controlled redox potential - Simpler product isolation - Simple monitoring of reaction progress 	<ul style="list-style-type: none"> - Incompatible with many biochemical systems - Poor selectivity - Complex apparatus and procedures - Rapid fouling of electrode surfaces - Potential requirement mediating redox dyes or enzymes
Chemical	<ul style="list-style-type: none"> - Generally inexpensive and commercially available reagents - No requirement for potentially expensive enzymes - High redox potentials 	<ul style="list-style-type: none"> - Limited compatibility with biochemical systems - Complexity of product isolation - Low desired product yields - Low co-factor recycling turnover number - Slow reaction rate
Photochemical	<ul style="list-style-type: none"> - Often non-stoichiometric regenerating reagents - No requirement for added enzymes 	<ul style="list-style-type: none"> - Complex apparatus - Limited compatibility with biochemical systems - Requirement for photo-sensitizers and redox dyes resulting in product contamination

Table 1.2: Examples of enzyme coupled co-factor recycling reactions.

Recycling system	Reactor type	TTN	Reference
<p>(a)</p>	Enzymatic Membrane Bioreactor	1200-62000	Lin <i>et al.</i> , 1997
<p>(b)</p>	Soluble enzyme in repetitive batch	160	Mertens <i>et al.</i> , 2003
<p>(c)</p>	Soluble enzyme in batch	> 1000	Pollard <i>et al.</i> , 2006
<p>(d)</p>	Co-immobilisation on nanoparticles in batch	18 000	Liu <i>et al.</i> , (2009)

TTN - total turnover number of co-factor; GDH-glucose dehydrogenase, GluDH-glutamate dehydrogenase; PDH-phenylalanine dehydrogenase; FDH-formate dehydrogenase; ADH-alcohol dehydrogenase; LDH-lactate dehydrogenase.

1.5 Co-factor recycling in immobilised enzyme-coupled systems

Enzyme immobilisation onto solid supports does not only improve stability, it also provides a convenient method to separate and re-use the enzyme; this can effectively reduce the cost of the enzyme and can thereby assist in realising their applications (Mateo *et al.*, 2007). This extends to enzymatic co-factor recycling systems (Polizzi *et al.*, 2007). El-Zahab *et al.*, 2004 co-immobilised LDH, GDH and the reduced NADH co-factor using four different spacers, glutaraldehyde, polyethylene-glycol (PEG, Mr 550 and 10 000 kDa) and PEG-BSA-PEG, in nanoporous silica glass supports. The coupled reactions were achieved with

regeneration of NADH/NAD⁺ following the reaction depicted in Fig. 1.3. This indicated the successful shuttling of the immobilised co-factor between the two enzymes where the reaction rates generally increased as longer spacers were used, in particular during the use of PEG-BSA-PEG, with a recycling rate of NADH of up to 102 h⁻¹ total turnover number (TTN) in a 1 litre reaction.

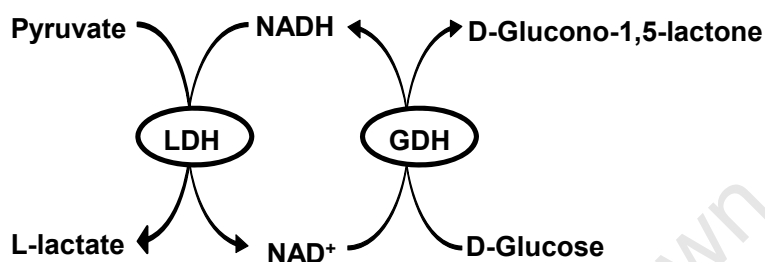


Figure 1.3: Diagram depicting the catalytic activity in the nano-structured co-factor recycling system using LDH and GDH (Adapted from El-Zahab *et al.*, 2004).

Mak *et al.*, 2003 developed an amperometric bi-enzyme sensor for determination of formate; immobilising FDH and salicylate hydroxylase and polyethylene glycol (PEG) linked co-factor NADH (PEG-NADH) onto a poly-(vinyl alcohol) matrix in front of a Clark-oxygen electrode. The reaction was monitored by oxygen consumption (Fig. 1.4). The FDH catalysed the conversion of formate to carbon dioxide with the concomitant utilisation of PEG-NAD⁺ to PEG-NADH and salicylate hydroxylase successfully oxidised the co-factor back to PEG-NAD⁺ using sodium salicylate and oxygen as substrates. This method is however prone to product inhibition as a direct result of accumulation of product(s) behind the membrane, leading to a decrease in biosensor response (Albery & Barlett, 1985; Inovan *et al.*, 2000).

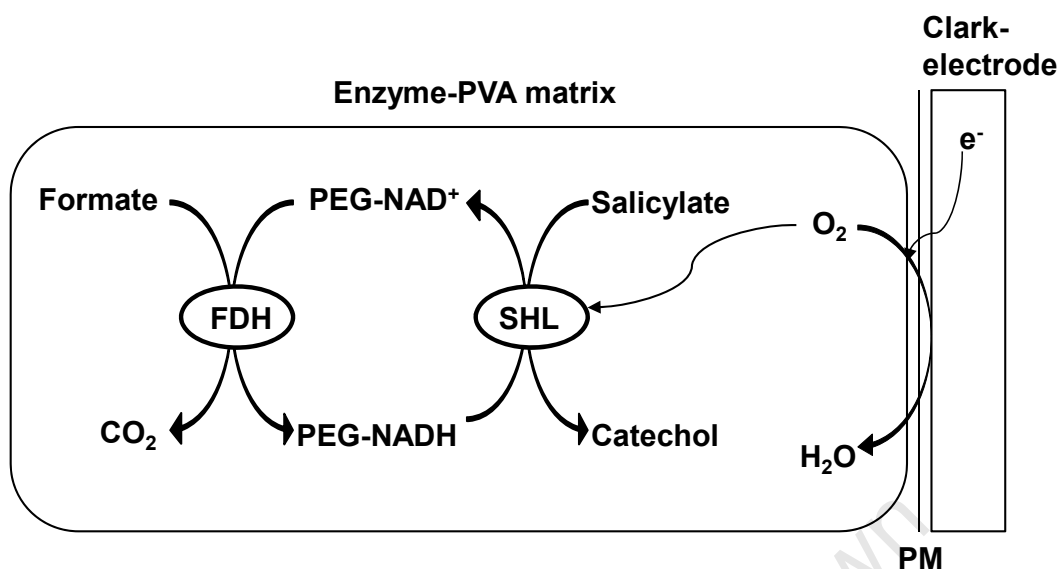


Figure 1.4: FDH and salicylate hydroxylase based biosensor for formate in a co-immobilised system with concomitant regeneration of the co-factor NADH/NAD⁺ (PM - polyethylene membrane; PVA - poly-(vinyl alcohol)) (Adapted from Mak *et al.*, 2003).

1.6 ReSyn™ polymeric immobilisation support

Jordaan *et al.*, 2009a developed a proprietary protein immobilisation support which involves the formation of a loosely-linked polymeric strands using a water-in-oil bi-emulsion process. The polymeric strands are formed by reacting a highly branched polymer, polyethyleneimine (PEI), and the water-soluble 5-carbon linear, dialdehyde crosslinking agent glutaraldehyde. The poly-cationic nature of PEI (high density of primary amine functional groups) has resulted in its use in a variety of applications such as tissue culture (Vancha *et al.*, 2004), fabrication of biosensors (Reybier *et al.*, 2002) and enzyme stabilisation during immobilisation (Obon, *et al.*, 1997). Glutaraldehyde has found applications in microscopy, leather tanning, enzyme stabilisation, chemical sterilisation and several other biomedical and pharmaceutical applications. It is generally understood that glutaraldehyde is more efficient than other aldehyde chemicals in generating thermally and chemically stable cross-links (Migneault *et al.*, 2004). The resulting cross-linked polymer network, ReSyn™ beads, has interstitial openings which allow for a high protein binding capacity (Jordaan *et al.*, 2009a). The degree of crosslinking between the aldehyde (glutaraldehyde) and amine (PEI) can be controlled by

changing the pH, thereby controlling the protonation of the amine groups (Wang *et al.*, 2006). It is further possible to entrap adjuncts such as co-factors within the matrix (Jordaan *et al.*, 2009a). This provides the opportunity to develop co-factor recycling systems (Jordaan *et al.*, 2009a).

1.7 Biomedical application of enzymes

The use of enzymes in medical applications has been less extensive compared to other types of industrial applications (Costa *et al.*, 2004). The application of enzymes in medicine has been successful in the extracellular removal of toxic substances, diagnosis using biosensors and the treatment of life-threatening blood disorders such as prevention and removal of blood clots (Kato *et al.*, 2001) and dissolution of blood clots (Torchilin, 1987). The use of biomaterials combined with biomolecules, such as enzymes, may yield biologically functional systems which could be used for biomedical applications (Costa *et al.*, 2004).

1.7.1 Biosensors and diagnostic assays

Diagnosis using enzymes is of major importance in monitoring progress after therapy, recovery following surgery, detecting transplant rejection or for detecting metabolites in serum or urine involved in various medical conditions (Costa *et al.*, 2004). The enzymes may be used in test strips, ELISA, biosensors and autoanalysers (Costa *et al.*, 2004). A number of oxidoreductases applied in the development of biosensors include; NADH oxidase in an amperometric NADH biosensor (Serban & El Murr, 2006), lactate dehydrogenase (LDH) for a lactate sensor (Rahman *et al.*, 2009), GDH for monitoring glucose (D'Costa *et al.*, 1986; Silber *et al.*, 1996) and FDH in detecting formate (Mak *et al.*, 2003). Most of the NADH/NAD⁺ dependent enzymes used in these amperometric quantification biosensors or bioassays are based on the theory that the direct oxidation of NADH at the electrode surface can be used as a detection reaction. However, this has proved to be inappropriate in practise as the high anodic potential induces interference and electrode poisoning in most cases (Serban & El Murr, 2006). Serban & El Murr, 2006 proposed a biosensor system which used NADH oxidase

to oxidise NADH in the presence of redox mediators. The enzymes, lactate dehydrogenase and glutamate dehydrogenase (GluDH), were used to prove this concept. The presence of NADH oxidase resulted in the rapid regeneration of the oxidised co-factor which shifted the equilibrium towards the pyruvate and 2-oxoglutarate formation from the LDH and GluDH respectively (Fig. 1.5). These kinetic conditions were considered extremely attractive as they allowed a highly sensitive measurement of the substrates, coupled with effective recycling of the co-factor. This demonstrated that the analytical system could function without the concomitant deterioration of the electrode.

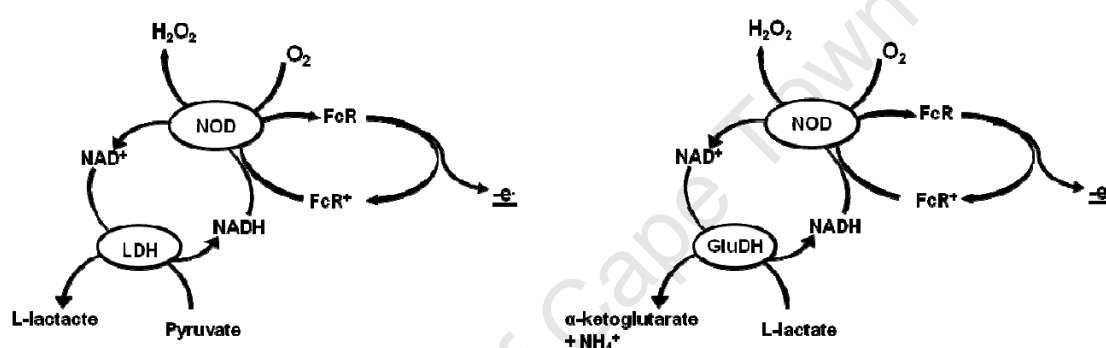


Figure 1.5: Schematic diagram depicting the biosensor using NADH oxidase (NOD) in the presence of redox mediators ($\text{FcR} - \text{FcR}^+$) with lactate dehydrogenase (LDH) and glutamate dehydrogenase (GluDH) for detecting lactate and glutamate respectively.

1.7.2 Therapeutics

Enzyme therapy is used in treating various conditions such as inborn metabolic disorders which result from the absence or malfunctioning of a naturally occurring enzyme, or in the removal of toxic compounds from blood or during cancer treatment (Costa *et al.*, 2004). For example, proline deficiency, a multisystemic hereditary disorder which in its severe form may result in imidodipeptiduria (a result of elevated levels of proline-containing dipeptides in the urine resulting in impaired development), is treated using enzyme replacement therapy (Genta *et al.*, 2001). Several other medical conditions caused by enzyme malfunction are currently being investigated e.g. Glucose-6-phosphate dehydrogenase (G6PDH). G6PDH is the sole supplier of the co-factor NADPH in red blood cells, and this co-

factor plays an important role in protecting the cells against oxidative stress (Costa *et al.*, 2004). The NADPH maintains glutathione in its reduced form, and reduced glutathione serves as an oxidative metabolite scavenger. Cells deficient in G6PDH undergo rapid haemolysis under oxidative stress (Zaitseva *et al.*, 2000). Immobilisation of G6PDH has previously been reported in an attempt to develop a therapeutic method for this condition, the highest operational stability was reported for carbodiimide activated polyacrylamide beads (Kotorman *et al.*, 1994).

There is interest in the administration of enzymes in the treatment of cancer. An example of such an enzyme is hyaluronidase which was shown to have intrinsic anticancer properties (St Croix *et al.*, 1998; Lin & Stern, 2001). The use of the enzyme reversed intrinsic multicellular drug resistance in compact EMT-6 tumor spheroids, the anti-adhesive effect of the enzyme rendered the tumours susceptible again to chemotherapy and cytotoxic drugs, therefore, acting as a non-toxic chemosensitizer (St Croix *et al.*, 1998).

1.8 Proposed co-factor recycling system

The aforementioned limitations on enzymes requiring co-factors have prevented the realisation of applications. It is evident that co-factor recycling systems that allow for the concomitant recovery of the enzyme can have an impact in realising these biocatalytic applications, while efficient co-factor recycling systems may further be useful for biomedical applications. Potential benefits of constructing an efficient co-factor recycling system would be the regeneration, recovery and continuous use of not only the catalyst but the expensive co-factor (Liu & Wang, 2007). This project serves to provide proof-of-concept for the preparation of an immobilised bi-enzymatic NADH/ NAD⁺ co-factor recycling system using the model enzymes, NADH oxidase (E.C. 1.6.3.1) and glucose dehydrogenase (E.C. 1.1.1.47).

The proposed system, shown in Fig.1.6, involves molecular oxygen being converted to water by NADH oxidase (NOD) with the concomitant oxidation of the polyethyleneglycol-linked NADH (PEG-NADH) to PEG-NAD⁺. Glucose dehydrogenase (GDH) will then recycle the co-factor back to its reduced form while converting D(+)-glucose to β-D-glucono-1,5-lactone. Both enzymes are of interest in biocatalytic co-factor regeneration along with their stabilisation through immobilisation is of further interest.

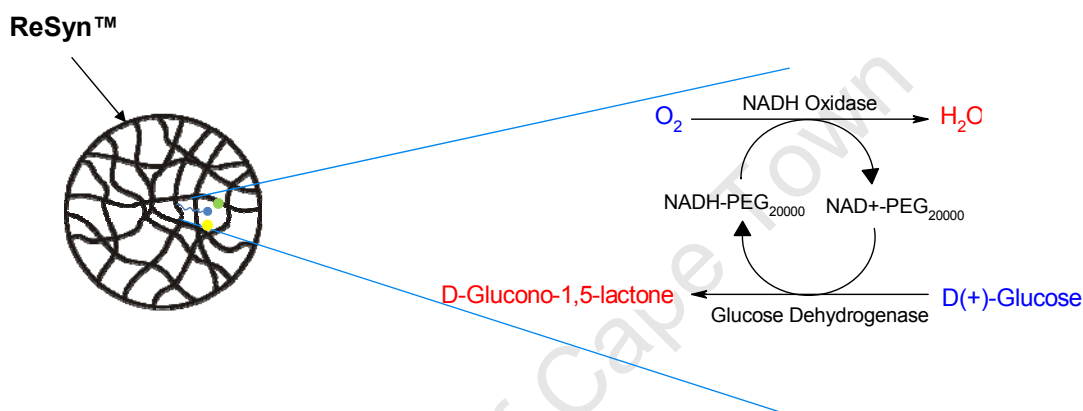


Figure 1.6: Diagram depicting the proposed construct as proof-of-concept for an enzyme-coupled ReSyn™-co-factor recycling system. The forward reaction is driven by the formation of water (H₂O) from molecular oxygen (O₂) resulting in the oxidation of the polyethyleneglycol-linked co-factor (PEG-NADH) using NADH oxidase. The reverse reaction involves D(+)-glucose being converted to D-glucono-1,5-lactone using glucose dehydrogenase results in the co-factor being reduced back to its original form.

1.9 Proposed ROS generation permutation of this system

Reactive oxygen species (ROS) are oxidising compounds which include superoxide radicals (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radicals (OH•) and other related species. These species are ubiquitous in nature and result from the incomplete reduction of oxygen during respiration, exposure to radiation or oxidative chemicals (Kong *et al.*, 2000). The unpaired electrons in the outer orbit of free radicals can react with biological cellular structures such as polyunsaturated membrane lipids, DNA and amino acids. Such reactions can lead to cell dysfunction, transformation and cell death. This occurs as a direct result of

the disintegration of the membranes, DNA damage and/or enzyme deactivation (Kong *et al.*, 2000). Since ROS is generically biocidal; parasites, pathogens and other cellular diseases have shown sensitivity to these chemical species. This has resulted in the development of drugs having reactive oxygen functionality. The susceptible disease causing agents include the *Plasmodium* parasite, the causal agent of malaria (Clark & Hunt, 1983; Postma *et al.*, 1996; Greve *et al.*, 1999); *Trypanosoma* the causal agent of the African sleeping sickness (Flohé *et al.*, 1999); the pathogen *Mycobacterium*, the causal agent of tuberculosis (Sherman *et al.*, 1995; Kwiatkowska *et al.*, 2007); and the cellular disease cancer is also susceptible to ROS to some degree (Yoshikawa *et al.*, 1995; Kong & Lillehei, 1998; Kong *et al.*, 2000). The biocidal effect of ROS on these diseases may provide an opportunity to exploit these natural metabolites and this results' in ROS balance levels to be elevated; similar to the effect of ROS based drugs (Trachootham *et al.*, 2006).

A proposed ROS generating permutation of this system would be of interest for biomedical application due to the biocidal activity of the reaction products. The enzyme NOD can be replaced with NADPH oxidase, an enzyme found in macrophages that is involved in the production of ROS as a natural defense response to infective agents or as a defense against cancerous cells (Kong *et al.*, 2000). This system is outlined in Fig. 1.7.

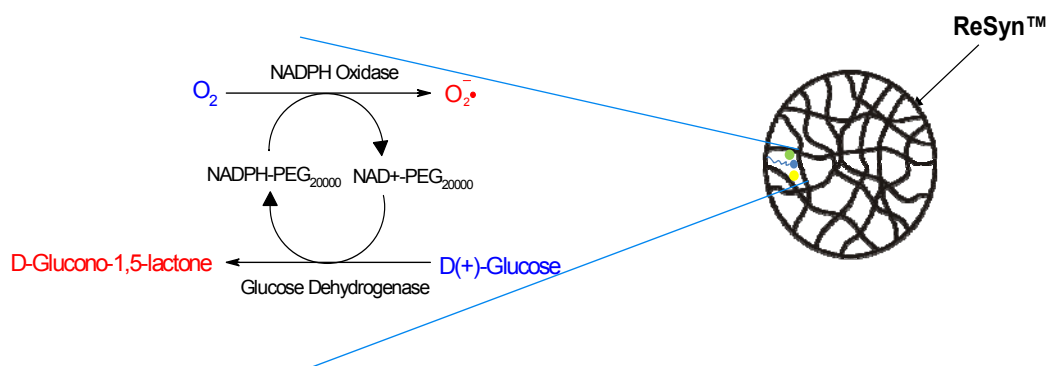


Figure 1.7: Diagram depicting the proposed construct of the enzyme-coupled ReSyn™-co-factor recycling system which generates reactive oxygen species (ROS) to be considered for therapeutic applications. The forward reaction is driven by the formation of superoxide ($O_2^{\bullet-}$) from molecular oxygen (O_2) resulting in the oxidation of the polyethyleneglycol-linked co-factor (PEG-NADPH) using NADPH oxidase. The reverse reaction involves D(+)-glucose being converted to D-glucono-1,5-lactone using glucose dehydrogenase results in the co-factor being recycled back to PEG-NADPH.

This research investigated the optimisation of the ReSyn™ support for enzyme immobilisation, activity maintenance and stability of the immobilised enzymes towards the development of the enzyme-coupled co-factor recycling system.

1.10 Research hypothesis

A self-contained NADH/NAD⁺ co-factor recycling system can be constructed using support based co-immobilisation of the enzymes, GDH and NOD, with co-entrapment of an enlarged PEGylated co-factor, NADH.

1.11 Objectives

- Preparation and evaluation of ReSyn™ particles of different properties for protein immobilisation.
- Individual immobilisation and characterisation of GDH and NOD on the various ReSyn™ preparations to determine the most suited support for co-immobilisation

- Co-immobilisation of GDH and NOD and evaluation of activity for construction of final system
- Entrapment of co-factor in polymer matrix along with co-immobilised enzymes
- Evaluation of co-factor recycling system to attain proof-of-concept

University of Cape Town

Chapter 2

Preparation and Characterisation of ReSyn™ particles

2.1 Introduction

The interest in enzyme immobilisation has largely been driven by the benefits of immobilised enzymes with respect to their practical use in various applications (refer section 1.2.3) (Kim *et al.*, 2006). Even though enzymes are highly selective and efficient in their processes, in soluble free form they are generally unstable thus limiting their application. Enzyme immobilisation offers a solution by providing a support matrix which enhances stability, allows repeated use, simplified separation from reaction mixtures and prevention of enzyme contamination in the reaction product (Wang & Caruso, 2005).

The challenge in developing a successful immobilisation support lies in the characteristics of the support and how it affects the enzyme upon immobilisation. Lack of sufficient surface area for immobilisation and the leaching of bound enzyme are some of the setbacks associated with various immobilisation supports (Cao *et al.*, 2003). This results in a low binding capacity and potential difficulties in recovery and down-stream processing. ReSyn™ technology addresses some of these obstacles hindering enzyme immobilisation.

ReSyn™ is a polymeric support produced using an emulsion based technique. The polymer is comprised of strands of PEI cross-linked by means of glutaraldehyde, a cross-linking agent. The degree of crosslinking can be controlled and thereby the interstitial openings and functional group density of the particles. The functional groups provide means for protein immobilisation (Jordaan *et al.*, 2009a). The synthetic polymer, polyethyleneimine (PEI), involved in the manufacturing of the particles, has a high density of amine groups (Wang *et al.*, 2006). This feature ensures efficient crosslinking and a high degree of functional groups available to link biomolecules.

This chapter outlines the preparation, optimisation and characterisation of the ReSyn™ polymeric particles for protein immobilisation.

2.2 Objectives

- To prepare and characterise four different ReSyn™ particles to determine how different properties of the particles may affect protein immobilisation
- Use BSA as the standard protein to determine the maximum binding capacity of the particles

2.3 Materials and Methods

2.3.1 Reagents

Mineral Oil; Glutaraldehyde (25% aqueous solution; Grade II); Polyethyleneimine (PEI) (50% aqueous solution, MW 750 kDa) specified to have 25% primary, 50% secondary and 25% tertiary amino groups present in the purchased product; Sodium Chloride, Hydrochloric Acid (HCl; 38% fuming) and Triethylamine (TEA) were obtained from Sigma-Aldrich; Bovine Serum Albumin (fraction V; 98%) was purchased from Roth; Nonoxynol-4 (NP-4) was a gift from CHC chemical group (South Africa).

2.3.2 Manufacturing and optimisation

The ReSyn™ polymer particles were prepared using a water-in-oil bi-emulsion based method (Jordaan *et al.*, 2009a). In brief: 50 µl NP-4, the surfactant, was dissolved in 5 ml mineral oil and mixed using a vortex (IKA®) at maximum speed for 5 s. The first emulsion consisted of the above components and 200 µl of 10% PEI; in the second emulsion the PEI was replaced with 200 µl of 20% glutaraldehyde. Each solution was emulsified at maximum speed for 10 s using a vortex. The two emulsions were combined and allowed to crosslink for 60 min with

end-over-end mixing using an Intelli-Mixer at 60 rpm to form the micro-particles. The pH of the PEI was altered (pH adjusted with HCl) to pH 5; 7; and 9 and 11 to control the degree of crosslinking. These preparations were named A, B, C and D respectively (Prep A, B, C and D).

The particles were recovered by centrifugation at 3901xg for 5 min at 10°C using a swing-out bucket rotor. The oil was discarded and the particles were washed a minimum of 5 times with 30 ml of MilliQ H₂O (Millipore); vigorous shaking was applied to re-suspend the particles between the centrifugal (as above) recovery steps. Following the last wash the particles were re-suspended to a total volume of 5ml in MilliQ water. The preparation process is summarised diagrammatically in Fig. 2.1.

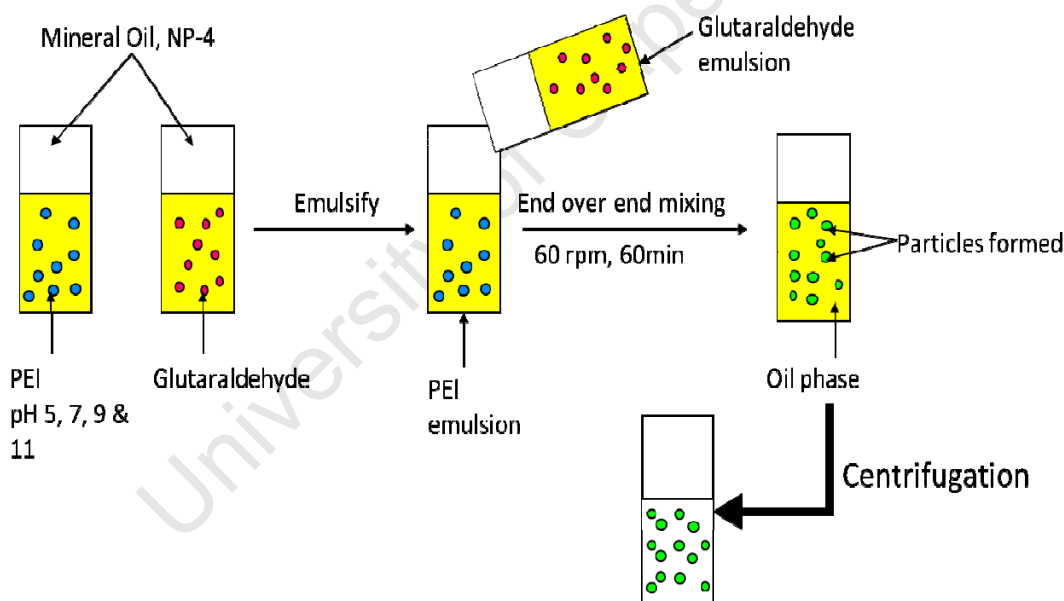


Figure 2.1: Schematic diagram depicting the preparation of ReSyn™ particles using a bi-emulsion method.

2.3.3 Dry-weight determination

The particles in samples of 1 ml in Eppendorf® tubes were spun at 6000 xg for 5 min on a bench-top centrifuge, excess water was discarded and the particles were freeze-dried (lyophilised) overnight with a shelf temperature of 0 °C and 0.3 mBar

(FreeZone® Triad™ Freeze Dry System, Labconco Corporation). The freeze dried particles were weighed to determine particle dry weight recovery from the various preparations.

2.3.4 Particle size distribution (PSD)

The particles in samples of 1 ml in 20% ethanol were analysed for particle size distribution (PSD) using a Malvern Mastersizer 2000 (Micron Scientific PTY LTD). The mean particle size analysis was determined under three conditions: pre-sonication; in-line sonication and post-sonication.

2.3.5 Protein binding capacity of the ReSyn™ particles

BSA was used as the standard protein to determine the protein binding capacity of the particle preparations. The particles were mixed to homogeneity using a vortex and 1 ml aliquots were pipetted into Eppendorf® tubes and were centrifuged at 6000xg for 5min; the pellets were washed three times in distilled water adjusted to pH 8.0, neutral and alkaline pH's are recommended for immobilisation of proteins on aldehyde and epoxide supports as the reactivity of the nucleophiles of lysine groups on proteins are improved at these pH's (Mateo *et al.*, 2007). The particles were recovered and resuspended using the same conditions as 2.3.2 above. Following the last wash, the water was discarded and the protein solution was loaded onto the various particle preparations. The binding capacity was evaluated by resuspension in 1 ml of a 20 mg.ml⁻¹ aqueous solution of BSA. The BSA was allowed to bind for 60 min with end-over-end mixing. The commonly used immobilisation buffer, 20 mM Triethylamine pH 8.0 (TEA), was further evaluated for protein binding using the same conditions mentioned above.

To quantify immobilised protein subsequent to binding, the samples were centrifuged for 5 min at 6000xg and the supernatant was assayed for unbound protein using UV-Vis spectroscopy at 280 nm on a micro-titre plate reader (Biotek, PowerWave HT) using UV transmissible plates (Greiner: PGRE655801).

Appropriate blanks consisting of all immobilisation components were used in each assay. To determine covalent binding; the supernatant from the samples was discarded and the particles with bound protein were rinsed twice with water and recovery performed by means of centrifugation as indicated above. The particles preparations were treated with 2 M NaCl to remove ionically bound protein therefore allowing the determination of covalently bound protein. All experimentation and assays were performed in triplicate.

2.4 Results

2.4.1 Particle preparation and characterisation

Four particle samples were prepared for evaluation. These preparations varied in the pH of the PEI used. The particles displayed different properties; initially following preparation the volume and colours of the particles varied as clearly visible in Fig. 2.2. The colour of the particles changed from light-orange to a darker orange-brown with the increase of the pH of the PEI, that is, Prep A to C respectively; ending with a pink colour for Prep D. The dry-weight, particles size distribution, and protein binding capacity of the particle preparations were determined.

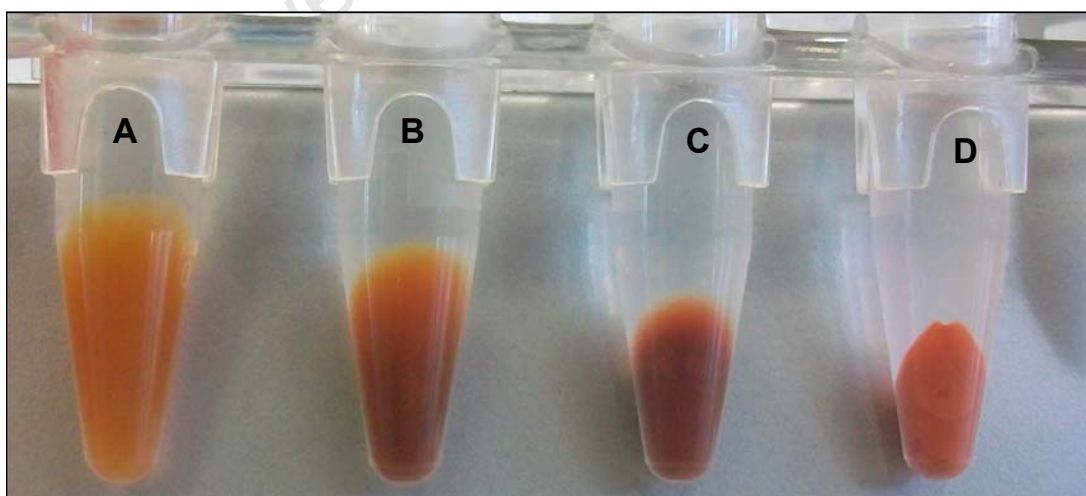


Figure 2.2: ReSyn™ particles prepared using polyethyleneimine (PEI) of various pH's. The volume of particles and particle colour intensity varied with pH of the PEI used for preparation. A, B, C and D refers to the different preparations used in this study.

2.4.2 Dry-weight determination

The yield (mass of particles recovered) from Prep A was the lower as compared to B, C and D, which did not show significant difference in their dry weights (Fig. 2.3). This may have been the result of the difficulty in handling of Prep A samples (loose particles were difficult to recover) or reduced incorporation of PEI due to reduced crosslinking.

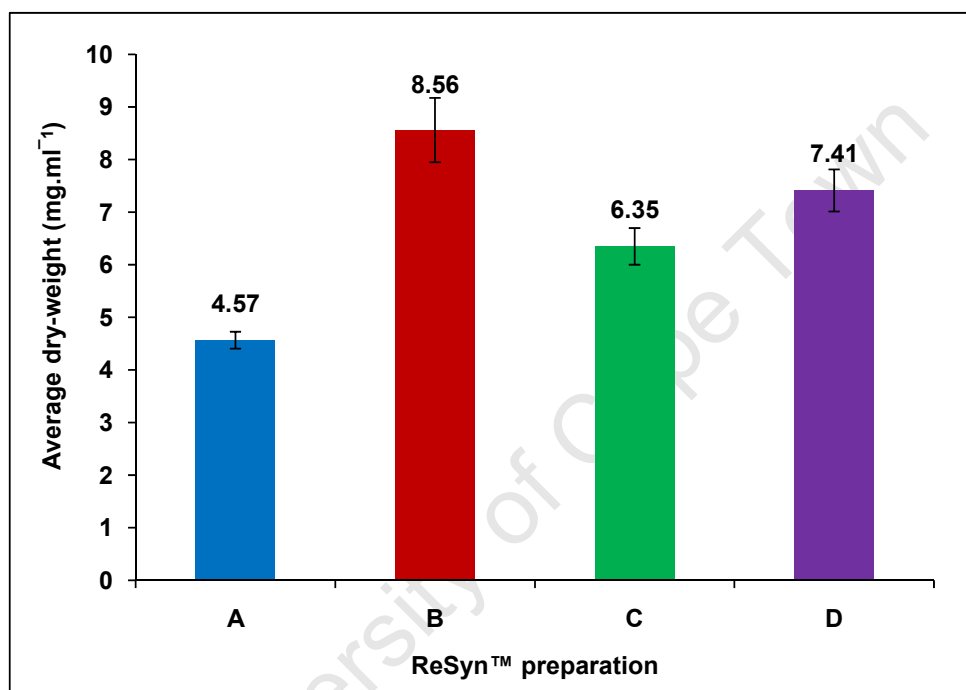


Figure 2.3: Average particle recovery (dry-weight) for various preparations. The data is presented as the mean \pm standard deviation for triplicate dry weight determinations.

2.4.3 Particle size distribution

Particle size distribution (PSD) was undertaken to determine whether there was difference in the size of particles prepared. The trend indicated a reduced particle size with increasing alkalinity of the PEI used to prepare the particles (Fig. 2.4). Prep A displayed the largest average particle size compared to the rest of the particles.

Particle size distribution determination was performed under 3 conditions. Pre-sonication, in-line sonication and post-sonication was performed to determine the presence of potential aggregate peaks. Fig. 2.5(a) indicates the aggregation peak (indicated by the arrow) that occurs with the particles over time and Fig. 2.5(b) shows the reduction of the peak following in-line sonication during measurement, indicating that the peak was a result of aggregated particles. Average particle size of non-aggregate peaks are reported in Table 2.1 below.

Prep C and D displayed a PSD ranging less than 10 μm whilst Prep A and B the average values were above this 10 μm (Fig. 2.6). All particle samples were subject to time-dependent aggregation. The general trend indicated more ready aggregation with more acidic pH of the preparation, i.e. Prep A aggregating more readily with while Prep D aggregating the least.

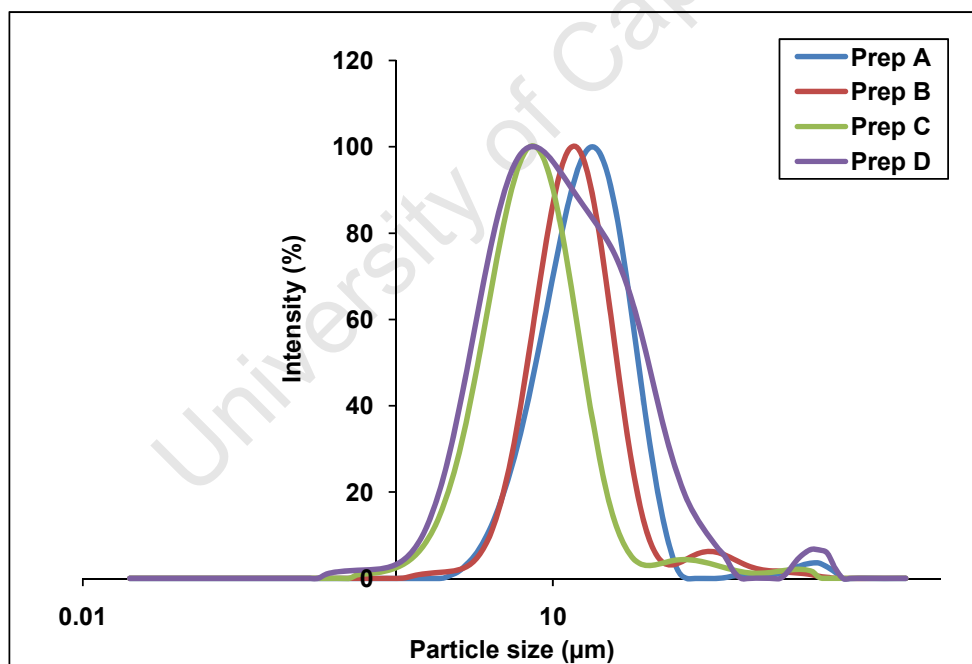


Figure 2.4: Diagram depicting the difference in size distribution of the various ReSyn™ particles manufactured using PEI of different pH. The analysis was done with in-line sonication using the Malvern Mastersizer 2000 (Micron Scientific PTY LTD). Prep A had a PSD averaging 20 μm , Prep B with 11, and C and D with 7 and 6.6 μm respectively.

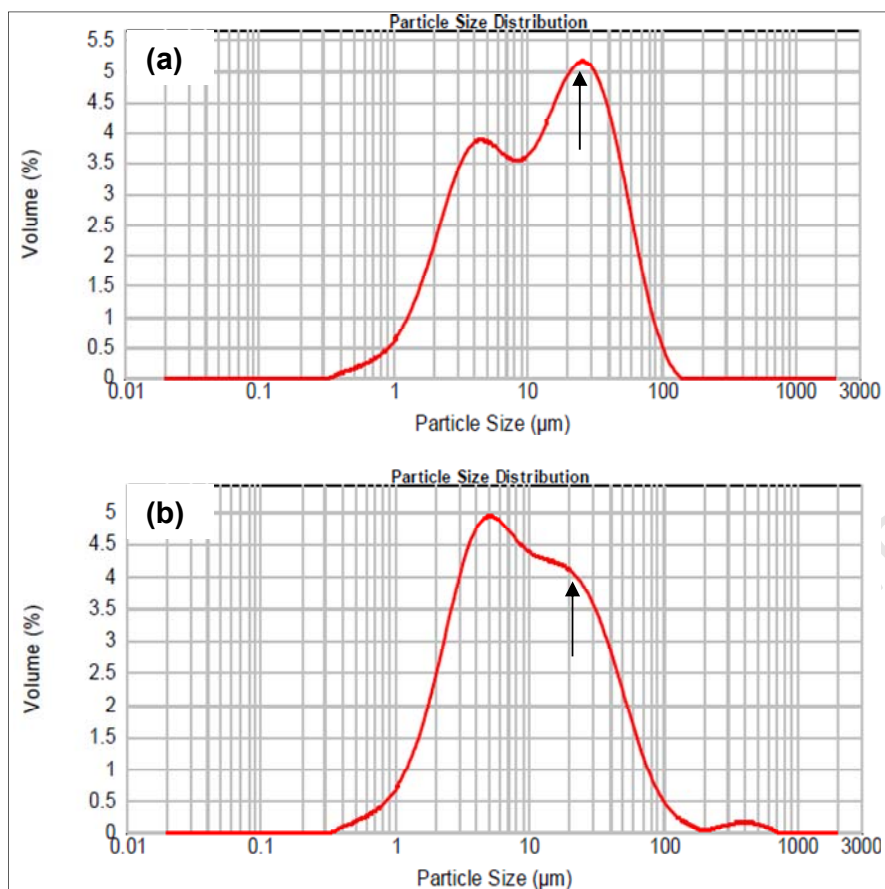


Figure 2.5: Examples of PSD curves showing the phenomenon of aggregation within the particles. (a) PSD determined pre-sonication, (b) PSD determined with in-line sonication. The aggregation peak is reduced with in-line sonication indicating the presence of aggregates. The PSD is for Prep D particles analysed 2 weeks after preparation.

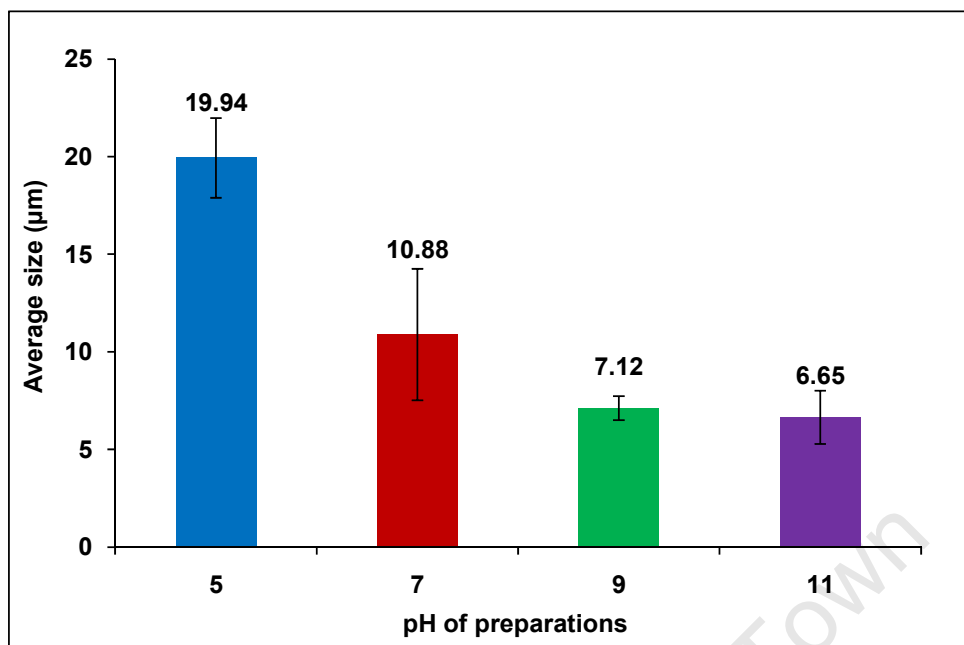


Figure 2.6: Average size distribution of the various ReSyn™ particles. The data is presented as the mean of triplicate data \pm standard deviation.

2.4.4 Protein binding capacity of ReSyn™ particles

The maximal binding capacity of the particles was determined using BSA as a standard protein (2.3.5). This was performed in both MilliQ H₂O and Triethylamine (TEA) buffer at pH 8.0. This pH was selected as the reactivity of the nucleophilic primary amines of the protein lysine residues with the available aldehydes within the particles increases with increased alkalinity (Mateo *et al.*, 2007). The results indicated the lower the degree of crosslinking in the polymer the higher the binding capacity

Table 2.1 shows results following washing with 2 M NaCl to determine covalently bound BSA on the particles. Prep A displayed the highest binding capacity in both MilliQ H₂O and TEA facilitated binding with 2.62 and 1.80 mg.mg⁻¹ binding respectively. Prep D displayed the lowest binding in both MilliQ H₂O and TEA with 0.29 and 0.14 mg.mg⁻¹ respectively. The binding capacity decreased with highly cross-linked particles.

Table 2.1: Averages of milligram covalently protein bound per milligram ReSyn™ using MilliQ H₂O and Triethylamine both at pH 8.0

ReSyn™ Preparation	Binding Capacity (mg.mg ⁻¹)	
	MilliQ H ₂ O	TEA
A	2.62 ± 0.07	1.80± 0.17
B	1.71 ± 0.02	0.92 ± 0.07
C	0.95 ± 0.04	0.56 ± 0.04
D	0.29± 0.03	0.14± 0.01

* The data is summarised and averaged from the raw data in Appendix 1A

Prep A and D were included for comparison to industrial support matrices (Fig. 2.7), representing the highest and lowest binding capacity achieved in this study. Reported literature values for immobilised protein include lipase on Eupergit® C and Eupergit® C 250L with binding capacities of 5.24×10^{-2} and 3.9×10^{-2} mg.mg⁻¹ respectively (Knezevic *et al.*, 2006). Illanes and colleagues (2010) used glyoxyl agarose small and large particles (GA-SP and GA-LP) for the immobilisation of penicillin G acylase and achieved 8.93×10^{-2} and 9.13×10^{-2} mg.mg⁻¹. Sepabeads EC-EA bound of 0.15 mg.mg⁻¹ of benoylformate dehydrogenase (BFD). The immobilisation of lipase on Sepabeads EC-EP achieved a 0.12 mg.mg⁻¹ (Hilterhaus *et al.*, 2008). The highest reported literature value for protein immobilisation appears to be Bimodal mesoporous silica (BMS) which showed a lysozyme binding capacity of 0.40 mg.mg⁻¹ (Wang & Caruso, 2005). This was achieved with a relatively small protein (16.6 kDa – lysozyme), a larger protein, peroxidase (44 kDa), was bound onto the BMS particles yielding 4.8×10^{-2} mg. mg⁻¹ (Wang & Caruso, 2005).

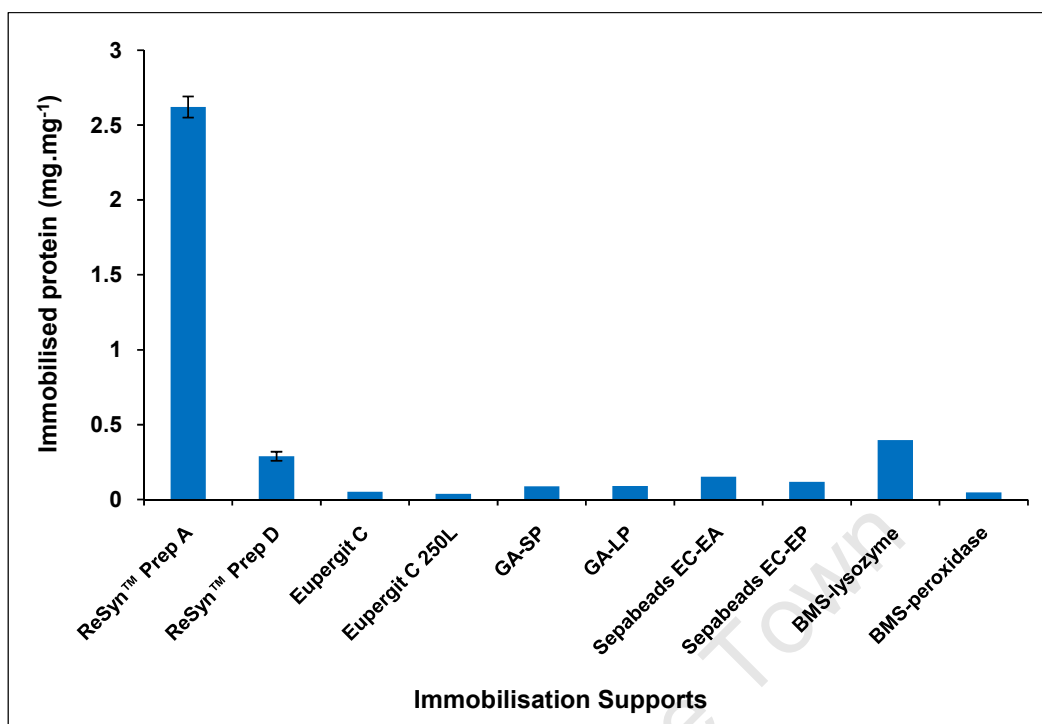


Figure 2.7: Protein binding capacities of various immobilisation supports in comparison with ReSyn™. (GA-SP - glyoxyl agarose small particle; GA-LP - glyoxyl agarose large particles; BMS - Bimodal mesoporous silica).

2.5 Discussion

The use of PEI at various pH's during the preparation resulted in polymer particles having different characteristics. Prep A had the highest protein binding capacity and the particles had a light-orange coloured loose pellet. This is likely due to the reduced crosslinking of the polymer network. Prep D had the lowest binding capacity, with the particles forming a compact orange-brown pellet. A general observed trend was noticed, i.e. the more acidic the pH of PEI the higher the protein binding capacity. Jordaan *et al.*, 2009a attributed this to the formation of a more loosely linked polymer matrix being formed as a result of reduced polymer crosslinking. The primary amine groups are more reactive at alkaline pH which enhances their reaction with aldehydes through the mechanism described earlier (Section 2.4). The colour of the matrix prepared at various pH values supports the theory of enhanced reaction (Ibrahim & Sharif, 2007). At acidic pH the equilibrium favours the formation of -NH_3^+ species over that of -NH_2 as a result of protonation

of the amine groups whereas at neutral and highly alkaline pH there is a high number of -NH_2 species (Wang *et al.*, 2006). With the glutaraldehyde added in excess it is assumed all the -NH_2 species are involved in the reaction, that is, in the formation of Schiff's base bonds. Experimental work in Dr Jordaan's laboratory has been done to verify whether the addition of extra glutaraldehyde will increase the functional group density of the particles and therefore binding capacity using pH 7.0 and 9.0 PEI (Jordaan *et al.*, 2009). The results from the work showed no increase indicating that this was indeed the case. The reaction scheme is indicated in Fig. 2.8 below.

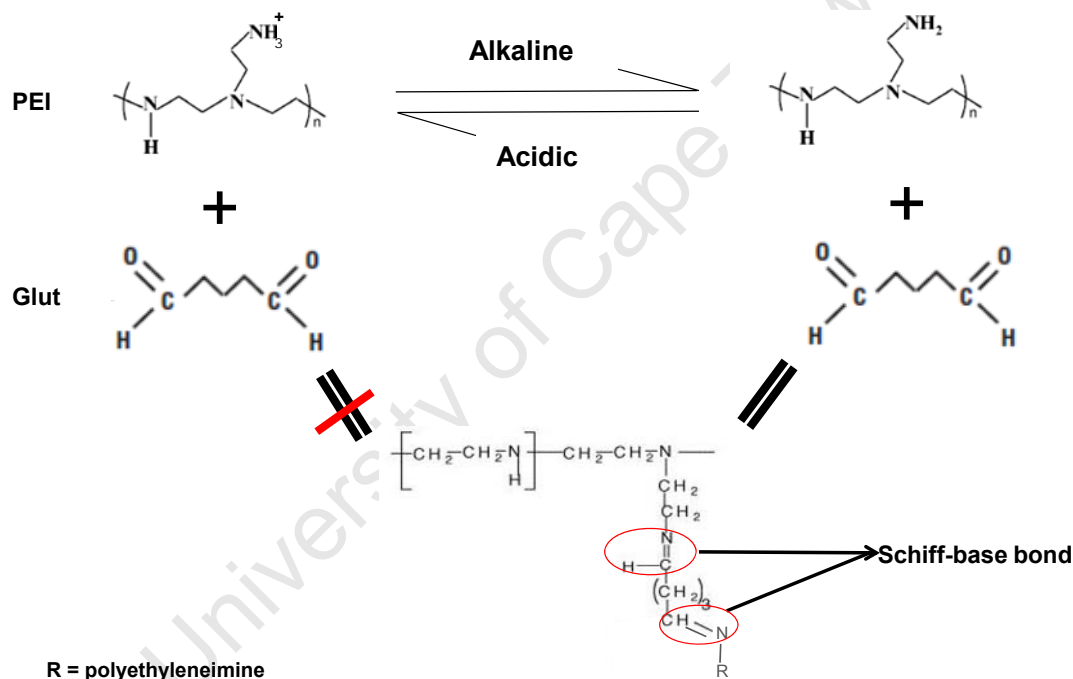


Figure 2.8: Diagram depicting the properties of PEI under different pH conditions. The reaction with aldehydes is not favoured at acidic conditions due to the protonation of the amine groups. (PEI - polyethylenimine, Glut - glutaraldehyde).

Particles prepared using PEI at pH 5 were difficult to recover as they did not compact during centrifugation. The centrifugation period of Prep A particles was extended to allow for recovery. Evidence of polymer/crosslinking agent were noted in the first step of washing and recovery (yellow-light orange coloured oil)

indicating incomplete incorporation of the polymer during crosslinking. These factors could have resulted in a reduced recovery for this particle preparation.

The protein binding capacity of the preparations was compared to reported values for commercial immobilisation supports (Fig. 2.7). The results indicated improvement in the binding capacity over alternate support technologies. The highest reported value to date is the immobilisation of invertase on a chemically surface modified silica gel with a reported capacity of $0.723 \text{ mg. mg}^{-1}$ (David *et al.*, 2006) while the highest binding capacity achieved here of 2.62 mg. mg^{-1} a 3.6 fold improvement. The high binding capacity can also be attributed to the high functional group density observed when compared to other immobilisation supports. From the work done in our lab (by Fezile Khumalo and Justin Jordaan) Prep B was reported to have a functional group density of $3.500 \text{ mmol.g}^{-1}$ and $1.147 \text{ mmol.g}^{-1}$ for Prep C. This is between 3.8 - 12X higher when compared to most supports such as Eupergit® which ranges between $\leq 0.3 \text{ mmol.g}^{-1}$ – 0.6 mmol.g^{-1} . High loading capacities of supports have been reported to increase the volumetric productivity (Li *et al.*, 2009). However increased enzyme loading has previously been reported to result in reduced productivity (Chae *et al.*, 1998; Persson *et al.*, 2000).

2.6 Conclusions

ReSyn™ particles were prepared under four different pH conditions. Each preparation varied with respect to their protein binding capacity. The general trend was that the more acidic the pH of the PEI used in the preparation, the higher the protein binding capacity. The inferred reduced crosslinking further led to an increase in the particle size distribution of the preparations.

The immobilisation capacity was detrimentally affected by a commonly used immobilisation buffer, i.e. Triethylamine. The use of this buffer will further be evaluated for enzyme activity maintenance.

The protein binding capacity of ReSyn™ appears to be superior to alternative protein immobilisation supports. The reproducibility of the preparations was suitable for enzyme immobilisation studies.

University of Cape Town

Chapter 3

Optimisation and Characterisation of ReSyn™ immobilised Glucose Dehydrogenase

3.1 Introduction

Dehydrogenase enzymes are capable of the stereospecific reduction of carbonyl groups for the production of various chiral compounds such as hydroxyl, amino acids or alcohols from prochiral precursors; these compounds are usually of high economic value (Hummel & Kula, 1989). These chemicals are used in the food and feed industries, or may serve as valuable building blocks in the synthesis of drugs, herbicides, and insecticides (Hummel & Kula, 1989). An important dehydrogenase, which has been applied in various industrial processes, is glucose dehydrogenase (GDH, E.C. 1.1.1.47). This enzyme catalyzes the oxidation of β -D(+)-glucose to β -D-glucono-1,5-lactone with the concomitant reduction of the co-factor NAD(P)^+ to NAD(P)H (Baron *et al.*, 1997; Baik *et al.*, 2003; Kataoka *et al.*, 1998; Manjon *et al.*, 2002). A specific example of a biocatalytic reaction is the asymmetric reduction of ethyl-4-chloro-3-oxobutanoate to (R)-4-chlor-3-hydroxybutanoate (Wong *et al.*, 1985). However, the use of GDH and other various dehydrogenases in these industrial applications is hindered by the fact that the enzymes requires the addition of stoichiometric amounts of the expensive co-factors which are not physically and permanently attached to the enzyme (Baik *et al.*, 2003, Leonida, 2001, Liu & Wang, 2007). This problem has partially been solved by the development of co-factor regenerating systems. A particularly favoured method is the bi-enzymatic method as enzymes are highly selective and efficient and this method also allows the coupling of more than one valuable chemical route (Liu & Wang, 2007) leading to economical viability. Such recycling systems have been developed through the microencapsulation of the enzymes and co-factor (Grunwald *et al.*, 1979; Wahl & Chang, 1987; Stengelin & Patel, 2000); immobilisation of both or immobilisation of the enzymes with entrapment of the co-factor (Yamazaki & Maeda, 1982; Parida *et al.*, 1992; Wang *et al.*, 2005). Recycling involving immobilisation of the enzyme of interest, will not only see GDH

regenerate the co-factor to its reduced form (NAD(P)H), it will also provide a platform to enhance the stability of GDH (Baron *et al.*, 1997) which is of importance when being applied in industrially.

There are few reported examples of the successful immobilisation of GDH. This may be due to the difficulties associated with immobilisation of multimeric enzymes. The enzyme may be easily distorted or denatured leading to significant loss of activity through subunit dissociation (Mateo *et al.*, 2007; Filho *et al.*, 2008; Bolivar *et al.*, 2009). This can be overcome through the use of supports which offer multipoint attachment (Mateo *et al.*, 2007). The polyethyleneimine support developed by Jordaan *et al.*, 2009a, may offer such a solution to the immobilisation of this enzyme.

This chapter investigates the immobilisation of GDH, on the proprietary polymer network, ReSyn™ with subsequent characterisation of the immobilised enzyme and physical properties including pH and temperature stabilisation.

3.2 Objectives

- To immobilise GDH on the various ReSyn™ preparations and characterise the changes in activity under various conditions with reference to the native enzyme

3.3 Materials and Methods

3.3.1 Reagents

Glucose dehydrogenase (E.C. 1.1.1.47) was purchased from Codexis (GDH 102 source organism: *Bacillus megaterium*); D(+) glucose, boric acid, citric acid, sodium phosphate dibasic, hydrochloric acid (HCl) and potassium hydroxide (KOH) were obtained from Sigma-Aldrich; β -nicotinamide adenine dinucleotide, free acid, grade I (NAD⁺) was obtained from Roche; Quick Start Bradford Protein Assay was obtained from BIORAD.

3.3.2 Enzyme preparation

GDH was desalted using PD-10 gel filtration columns (GE Healthcare) before immobilisation to remove possible contaminants that could interfere with immobilisation. This was done according to the manufacturers' protocol. In brief: The column was equilibrated with 25 ml of MilliQ H₂O (pH 8.0), 2.5 ml of a GDH suspension (5 mg.ml⁻¹) was applied to the column subsequently eluted with 3.5ml of the MilliQ H₂O. The sample was assayed for protein quantity and made to a final concentration of 1 mg.ml⁻¹. For buffered immobilisation, the PD-10 columns were equilibrated, and the protein eluted, in 20 mM TEA buffer pH 8.0.

3.3.3 Immobilisation of GDH

Aqueous, and TEA buffered, suspensions of GDH (1 mg.ml⁻¹ pH 8.0) were used for immobilisation on to pre-determined aliquots of particles as in section 2.3.5. These aliquots were approximated from the associated BSA binding capacities in 2.4.4; the GDH was loaded onto the particles in excess (20%) to ensure saturation of the particles and allowed for the maximum binding capacity for this enzyme to be achieved. Immobilisation was achieved by incubation of the particle-enzyme suspension for 60 min at 8 °C with end over end mixing at 25 rpm. The particles were recovered according to the procedure described in section 2.3.5. The supernatant was assayed for unbound protein using the Bradford assay (Bradford, 1976) following the product protocol for standard microplate assay recommended by Bio-Rad. The Bradford assay is based on the binding of the dye Coomassie Brilliant Blue G-250 to protein. This binding results in a shift in the absorption maximum of the dye, that is, from 465 to 595 nm. This increase in absorption at 595 nm is monitored (Bradford, 1976). A protein concentration standard curve was generated using GDH as the standard (Appendix 2A). Dye reagent, 250 µl, was added to 5 µl of sample and mixed thoroughly by pipetting. The assay samples were incubated at room temperature for 5 min before absorption spectroscopy at 595nm for non-immobilised protein quantification. The protein solution was replaced with water/buffer in the control assay. Covalent binding was determined as previously described in section 2.3.5 using 2 M NaCl. Immobilisation and quantification of GDH in the presence of TEA pH 8.0 was also done using the

procedure and conditions mentioned above. The standard curve used for quantification in TEA immobilisation is shown in Appendix 2B.

For subsequent assays and experiments the specific amount of covalently bound GDH for each particle preparation was used.

3.3.4 Free and immobilised GDH assays

Activity for both free and immobilised GDH was determined by following the kinetic reduction of NAD⁺ to NADH using spectrophotometric absorbance at 340 nm using a microtitre plate spectrophotometer (Biotek Instruments, PowerWave HT). The reduction of NAD⁺ to NADH requires the concomitant conversion of D-glucose to D-glucono-1,5-lactone. The assay reagent consisted of 1 mM NAD⁺ and 50 mM D (+) Glucose in 50 mM Tris-HCl pH 8.0; the assay was modified from the Sigma-Aldrich protocol for assaying GDH, BSA and triton X-100 were not used in the assay as they did not affect the assay and their effect on the particle assay was unknown. One GDH Unit was defined as the amount of enzyme required to reduce 1 μ mol of NAD⁺ per minute at 37 °C. The enzyme reactions contained 5 μ l of GDH solution with 195 μ l of assay reagent. An assay with standard concentrations of the enzyme, as described in the Sigma-Aldrich protocol, was done and the specific activity of the enzyme was calculated to be 116 U.mg⁻¹. All assays were performed in triplicate and are represented as the mean \pm standard deviation. The control assay contained water instead of the enzyme and was used as a blank for the assays. With immobilised GDH, the particles with bound enzyme were washed twice with MilliQ water and were made to a total volume of 100 μ l in 20 mM Tris-HCl pH 8.0. The immobilised enzyme preparations were diluted to within the linear kinetic range of the assay.

3.3.4.1 pH profiling

The pH profile assays for both free and immobilised GDH were performed using the same conditions and substrate concentrations as specified above. However, Tris-HCl pH 8.0 was replaced with a universal buffer containing 50 mM phosphate

(sodium phosphate dibasic) 50 mM Boric Acid, 33 mM Citric Acid and 50 mM Tris adjusted to pH values between 4-10 using HCl and KOH (Jordaan *et al.*, 2004).

3.3.5 Thermostability

Thermostability was determined by incubation of the free and immobilised GDH preparations from 50 to 65 °C with 5 °C increments and the activity was assayed initially at 15 min intervals as described in 3.3.4. The time-interval was extended for samples displaying high stability.

3.3.6 pH stability

pH stability was determined by incubation of the free and immobilised GDH preparations in 1X universal buffer pH 2.5, 3.0 and 3.5 (composition as above in 3.3.4) the activity was assayed initially at 10 min intervals as described in 3.3.4. The time-interval was extended for samples displaying high stability.

3.4 Results

3.4.1 Enzyme preparation

Desalting is a relatively non-destructive process useful for removal of low molecular weight contaminants that could potentially interfere with immobilisation, and as a method of buffer exchange. Following de-salting 6% of the loaded protein was not recovered. The recovered protein had the same specific activity as the starting material, indicating that no enzyme activity was lost during this procedure.

3.4.2 Immobilisation of GDH

Immobilisation of proteins in aqueous suspensions of MilliQ H₂O displayed the highest binding capacities for all preparations when compared to the binding

performed using TEA buffer (Table 3.1). In both MilliQ H₂O and TEA Prep A achieved the highest protein binding capacity while Prep D bound the least. This was in accordance to what was obtained with BSA (Chapter 2). A different trend was witnessed with Prep B and C where Prep C bound more enzyme than Prep B in both the presence of MilliQ water and TEA.

Table 3.1: Binding of GDH in the presence of MilliQ H₂O and Triethylamine on various ReSyn™ preparations.

ReSyn™ Preparation	Binding Capacity (mg.mg ⁻¹)	
	MilliQ H ₂ O	TEA
A	1.222 ± 0.003	1.141 ± 0.003
B	0.508 ± 0.002	0.354 ± 0.002
C	0.820 ± 0.002	0.419 ± 0.003
D	0.163 ± 0.030	0.098 ± 0.021

Raw data sufficient to demonstrate this binding capacity is included in Appendix 2B

3.4.3 Free and immobilised GDH assays

Of major importance when considering a technique for immobilisation is the maintenance of catalytic activity which was calculated as below:

$$\frac{\text{Activity of immobilized enzyme (U)}}{\text{Initial enzyme activity (U)}} \times 100\%$$

The maintenance in activity displayed by the four GDH preparations immobilised using MilliQ water ranged from 29.1 to 42.3%. The maintenance of activity in the presence of MilliQ water was higher than that displayed when using TEA, which achieved maintenance in activity of a low of 6.4 to a maximum of 10.3% (Fig. 3.1).

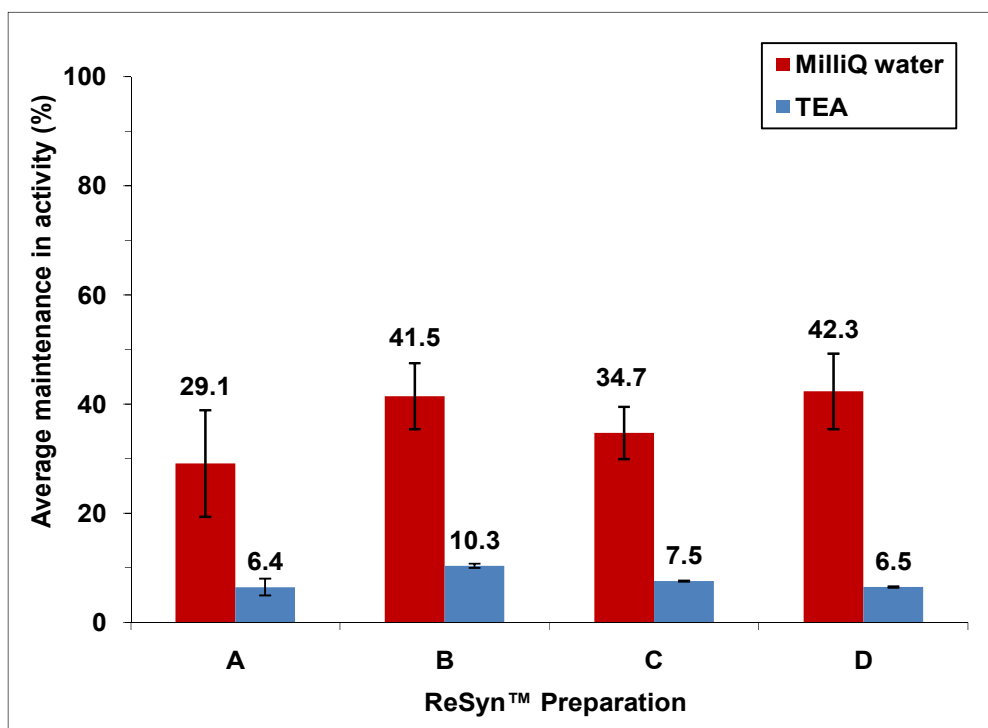


Figure 3.1: Average maintenance in activity displayed by GDH immobilised on various ReSyn™ preparations using MilliQ H₂O and TEA. The data is presented as the mean of triplicate data \pm the standard deviation.

The performance of the various preparations, along with the method used in the immobilisation of GDH, and specific activity, is summarised in Table 3.2 below. The activity of immobilised GDH (U.mg⁻¹ support) was directly proportional to the binding capacity; Prep A bound the most enzyme, resulting in the highest specific activity of 41.18 U.mg⁻¹. Prep B- and C-GDH did not show much difference in terms of the specific activity, whilst Prep D-GDH displayed the lowest activity.

Table 3.2: GDH activity following immobilisation on the various preparation of ReSyn™ using MilliQ H₂O.

ReSyn™ Preparation	GDH Activity (U.mg ⁻¹ support)		Specific Activity (U.mg ⁻¹ enzyme)	
	MilliQ H ₂ O	TEA	MilliQ H ₂ O	TEA
A	22.63	4.50	41.18	8.46
B	15.18	1.45	33.22	5.14
C	14.87	2.28	33.01	5.74
D	2.63	0.25	12.76	1.51

The specific activity of the free enzyme was 116 U.mg⁻¹.

TEA was not used for subsequent experimentation due to the low binding capacity, and low maintenance in enzyme activity. Also, Prep D immobilisation in the presence of water was not evaluated in subsequent experiments due to its low specific activity.

3.4.3.1 pH profiling

The pH profile for the enzyme and immobilised GDH is shown in Fig. 3.2. All preparations showed optimum activity at pH 8.0. GDH immobilised on Prep A and B exhibited a broader pH profile than compared to the free enzyme. This broadening is indicated by an increase in activity in the acidic range (pH 4.0-6.0) up to neutral pH 7.0. Prep A- and B-GDH displayed an improved activity at pH 4.0 with increases in activity from 28.2% for the free enzyme to 42.0 and 71.8% respectively. This equates to an increase in activity by a factor of 1.5 and 2.5-fold respectively. From pH 5 to 7, prep A and B displayed similar increases in activity, of approximately 2 fold. Prep C showed a similar profile to the free enzyme with no increased activity at any of the pH values tested.

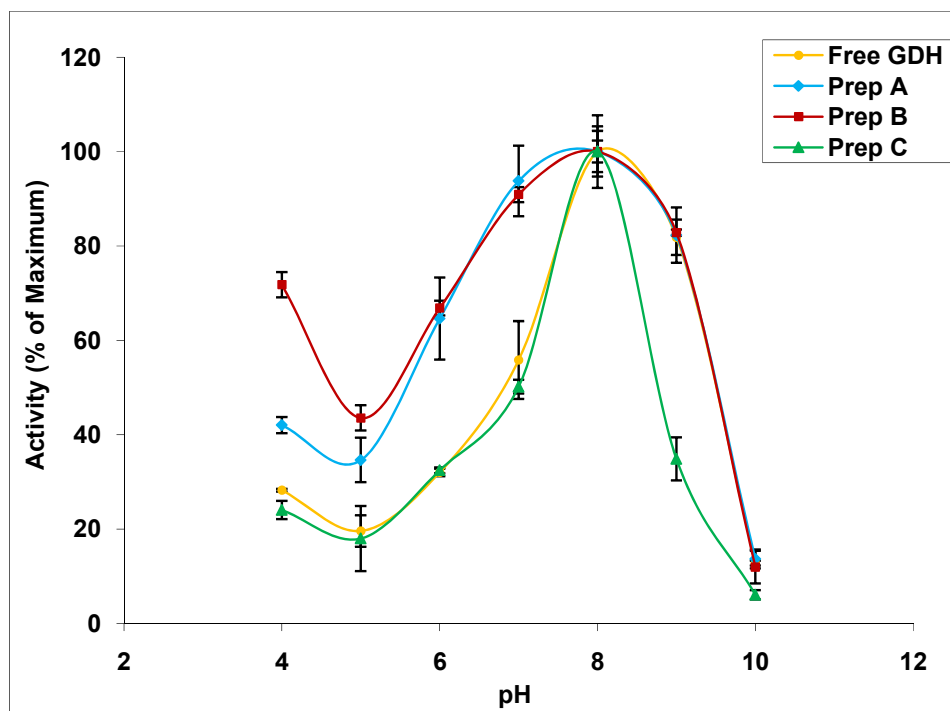
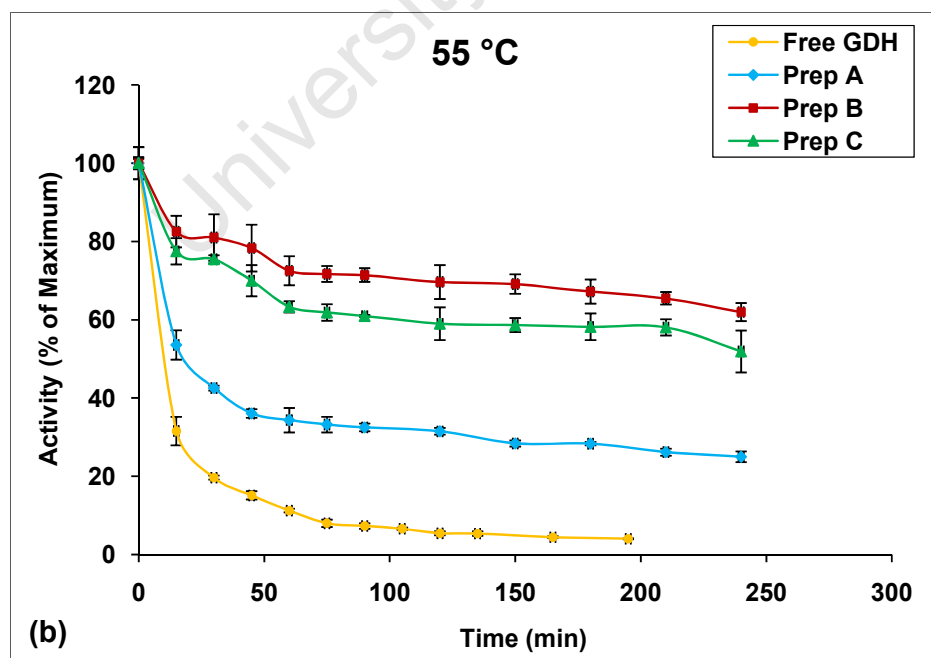
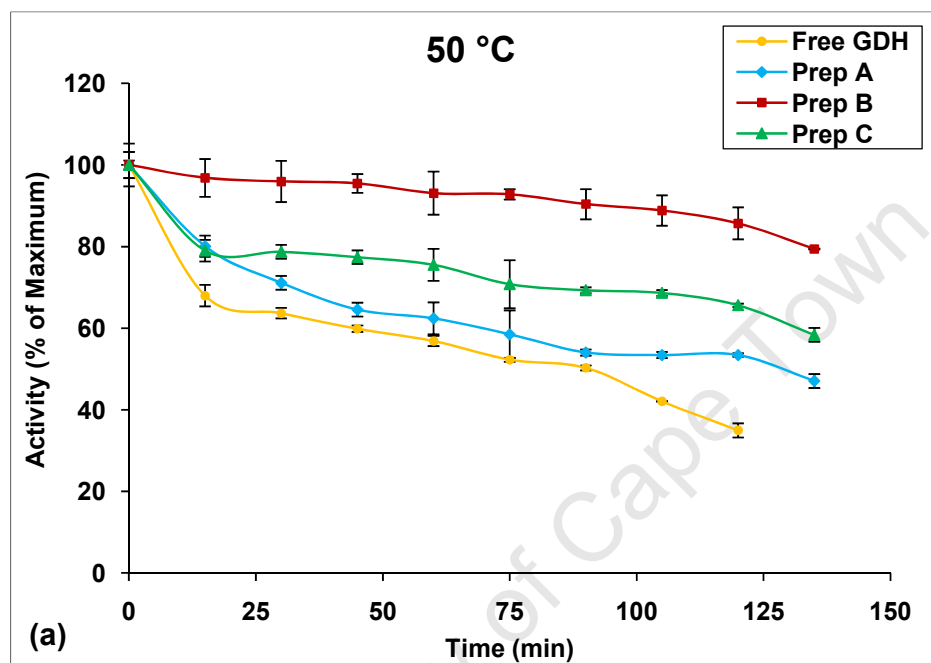


Figure 3.2: pH profile of free and immobilised GDH. The optimal pH of activity was displayed at pH 8.0. Prep A and B-GDH displayed a broader pH profile with increased activity over the acidic range. The data is presented as the mean of triplicate data \pm the standard deviation.

3.4.4 Thermostability

Immobilisation of enzymes has previously been reported to result in an improvement in the thermal stability of enzymes (López-Gallego *et al.*, 2005a; 2005b; Bolivar *et al.*, 2006b, 2009; Pedroche *et al.*, 2007; Filho *et al.*, 2008). Thermal stabilities of the various immobilised GDH preparations were compared to the free enzyme to quantify any improvement in the thermal stability. At 50°C free GDH had a half-life (t_{50} - defined as the time required to reach 50% activity) of 91 min. Prep A-GDH had a half-life of 128 min translating to a 1.4-fold improvement in stability (Fig. 3.3(a), Table 3.3) at the same temperature. The other preparations, Prep B and C-GDH exhibited higher stability at this temperature and did not reach 50% activity within the duration of the experiment (130 min). At elevated temperature incubations of 55, 60 and 65 °C the free enzyme reached the t_{50} at 11 min, 9 and 8 min respectively (Fig. 3.3(b-d); Table 3.3), repeatedly Prep B-GDH did not reach 50% activity during incubations 55 and

60 °C. Prep B-GDH clearly showed the highest improvement in thermal stabilisation. The improvement in stability at 65 °C compared to the free enzyme was 29-fold; while the improvement over Prep A-GDH and D-GDH was 19 and 17-fold respectively.



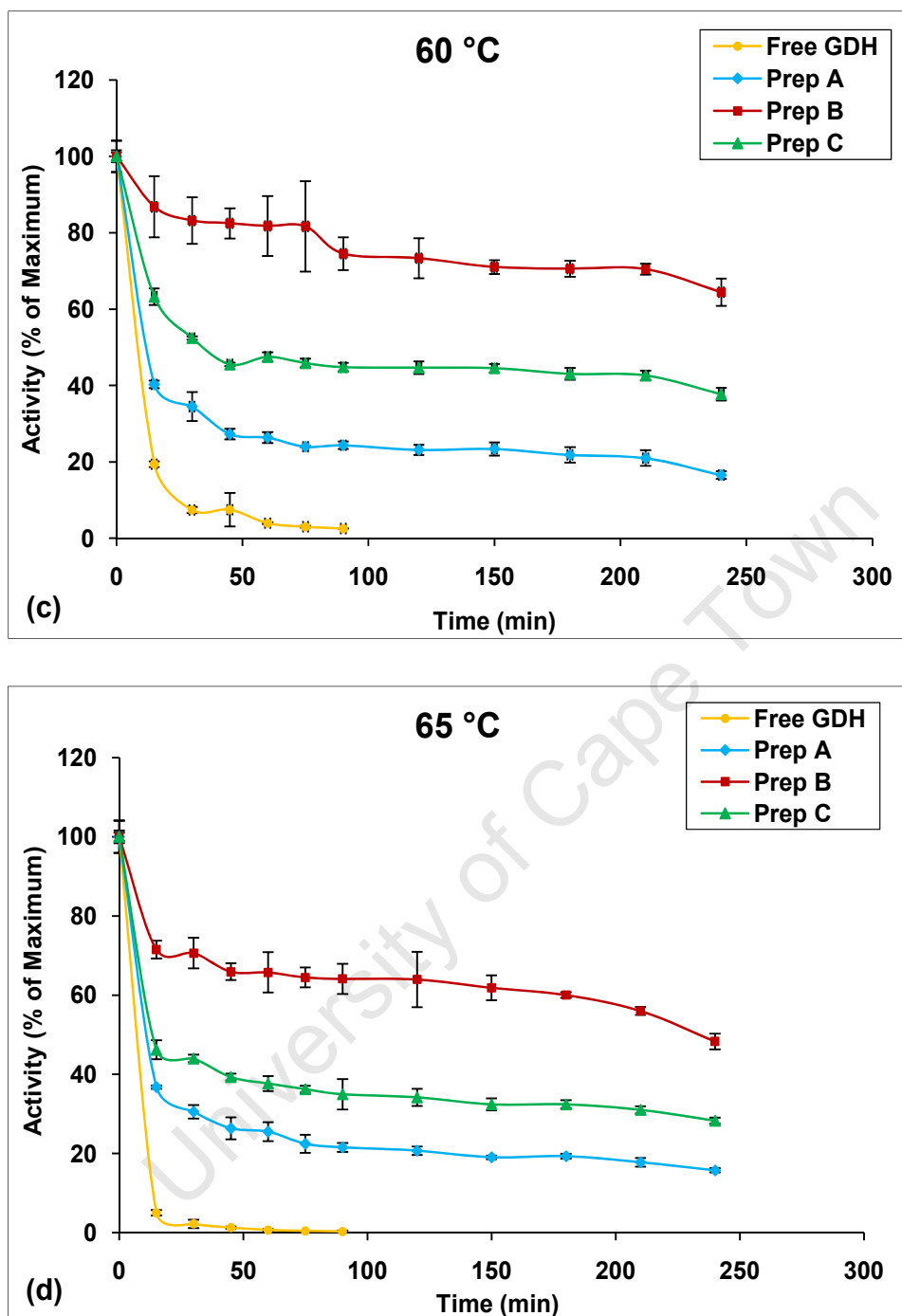


Figure 3.3: Thermal stability at (a) 50, (b) 55, (c) 60 and (d) 65 °C of GDH immobilised on the various particle preparations. Prep B-GDH was the most thermally stable over alternate preparations. The data is presented as the mean of triplicate data \pm the standard deviation.

Table 3.3: Thermal stability half-lives of the various preparations during incubation at elevated temperature.

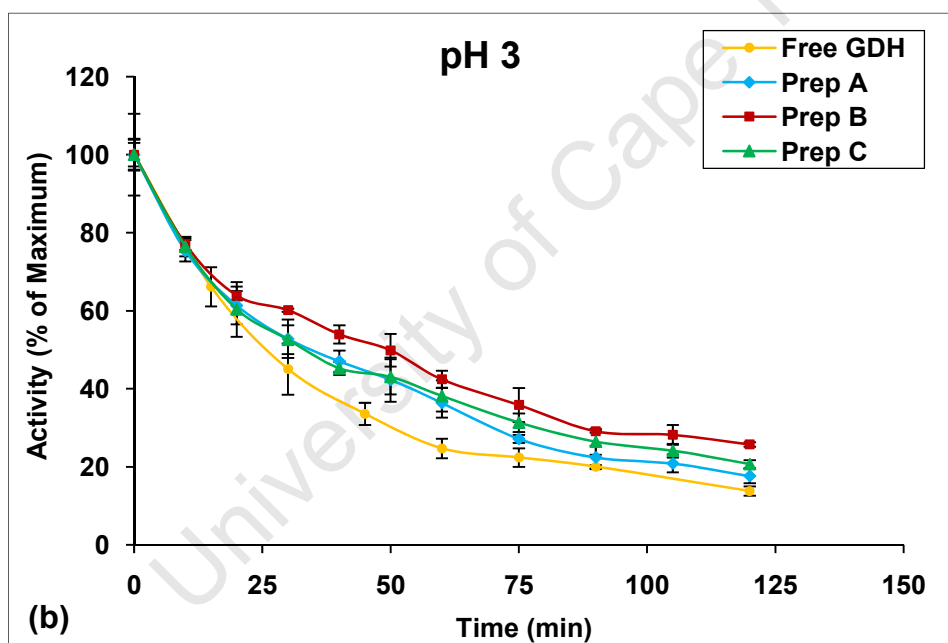
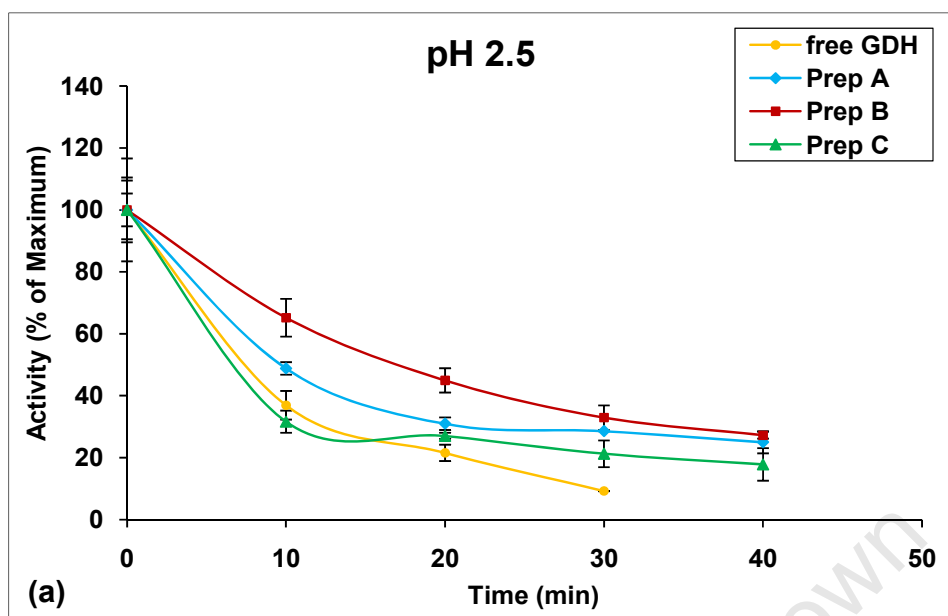
Temperature (°C)	Half-life (T_{50}) (min)			
	Free GDH	A	B	C
50	91	128	-	-
55	11	19	-	-
60	9	13	-	35
65	8	12	233	14

A, B and C indicate the different preparations for GDH immobilisation. The unspecified values are for those preparations where 50% activity was not reached during the time course of the experimental set. Enzyme deactivation was assumed to be of first order.

3.4.5 pH stability

The pH stabilities of GDH immobilised on the particles prepared under various conditions were compared to the free form of the enzyme (Fig. 3.4a-c). The stability was tested over the acidic range, that is, pH 2.5, 3.0 and 3.5. The half-lives determined for the free and immobilised GDH on the various preparations are summarised in Table 3.5. At pH 2.5 the immobilised enzyme preparations showed limited improvement in stability over the free enzyme (Fig. 3.4a). The most improved stability was displayed by prep B, with a half-life of 18 min, a 2.3-fold improvement over the free enzyme at this pH (8 min).

At pH 3.0 Prep A- and C-GDH had similar half-lives of 34 min, a limited improvement in stability of 1.3-fold over the free enzyme, while Prep B-GDH was the most stable with a fold improvement of 1.8. At pH 3.5 similar fold improvements of 1.9, 2.4 and 2.0 was witnessed with Prep A-, B- and C-GDH over the free enzyme respectively. Prep B displayed the highest stability in all scenarios tested.



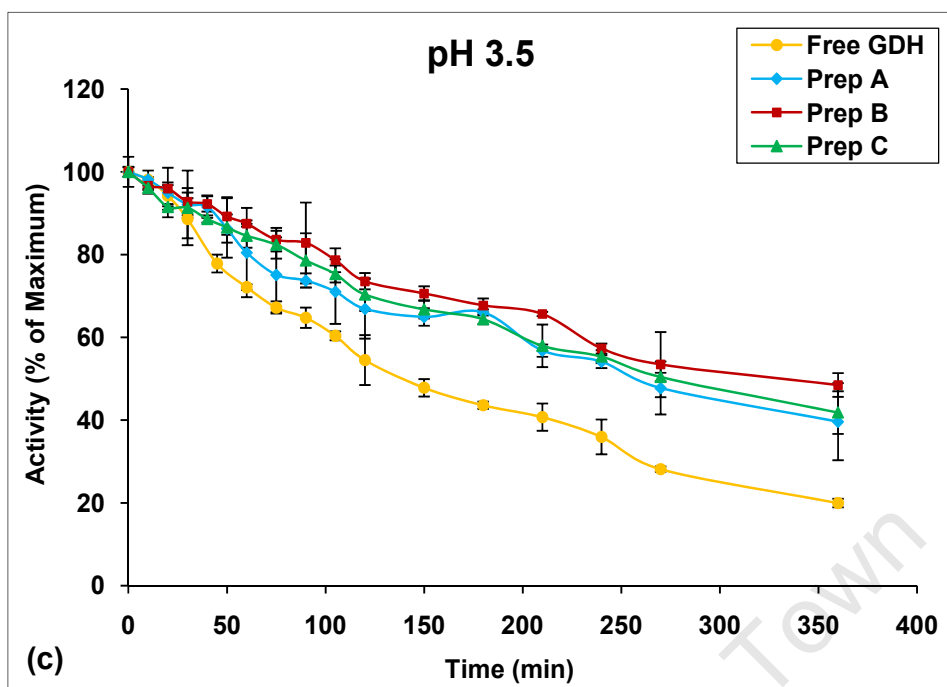


Figure 3.4: pH stability at (a) 2.5, (b) 3.0 and (c) 3.5 of GDH immobilised on the various particle preparations. Prep B-GDH was the most stable preparation over alternate preparation over this pH range. The data is presented as the mean of triplicate data \pm the standard deviation.

Table 3.4: Half-lives displayed by the free and immobilised GDH under the different pH conditions.

pH	Half-life (T_{50}) (min)			
	Free GDH	A	B	C
2.5	8	10	18	7
3	27	35	50	34
3.5	140	259	332	274

A, B and C indicate the different preparations used for GDH immobilisation. Enzyme deactivation was assumed to be of the first order.

3.5 Discussion

GDH was immobilised on various ReSyn™ preparations with subsequent characterisation to determine the most suitable support for GDH immobilisation. Research on the immobilisation of various multimeric enzymes has been conducted, with immobilisation via multipoint attachment and stabilisation of multimeric proteins being achieved. Examples of this include the hexameric α -galactosidase (Filho *et al.*, 2008) and the homodimer D-amino acid oxidase (López-Gallego *et al.*, 2005a). GDH immobilisation was achieved on all ReSyn™ particles tested. Prep A, as expected from pre-experimentation with BSA, bound the most protein. The binding achieved by each preparation was significantly lower than the amounts bound when BSA was used (Chapter 2), that is Prep A-BSA bound 2.62 mg.mg^{-1} and Prep A-GDH only bound 1.22 mg.mg^{-1} this trend was also witnessed with the other preparations. This is potentially due to the difference in the size of the protein (BSA – 66 kDa; GDH - 118 kDa; Jany *et al.*, 1984), where the larger protein molecules are potentially limited in penetrating the porous particle or a larger surface area is occupied by the protein on the particles limiting further immobilisation of other protein molecules. The reduced binding may have been due to the difference in reaction time for the binding between BSA and GDH; BSA (Chapter 2) was bound overnight with GDH binding done for an hour since when GDH was bound overnight $\geq 98\%$ of activity was lost. Sufficient reaction time allows the correct alignment of reactive groups between the enzyme and support (Mateo *et al.*, 2007). The observed increase in activity in the acidic range of the pH profile for the immobilised enzyme may be a result of the particles microenvironment (Mirzarakhmetova & Abdurazakova, 1998; Arica *et al.*, 2009; Jordaan *et al.* 2009b). The reduced crosslinking of the polymer backbone (chapter 2) results in Prep A and B being less interlinked leading to most of the enzyme being immobilised within the cross-linked particle, whilst Prep C is (higher degree of crosslinking) results in less enzyme penetration and thereby more exposure to the solution, and thereby more similar characteristics to the free enzyme.

The challenges in the immobilisation of multimeric enzymes may be a contributing factor to the lack of literature on the immobilisation of GDH considering its

importance for co-factor recycling. The only published work on the covalent immobilisation of GDH is on controlled pore silica which reported a maximum protein binding capacity of $4.4 \times 10^{-4} \text{ mg.mg}^{-1}$ (Baron *et al.*, 1997). This work reports a maximum of 1.22 mg.mg^{-1} . This equates to an over 2800 fold increase in the binding capacity for this protein. Immobilisation on the silica beads yielded a specific activity of 0.13 U.mg^{-1} , while Prep A, from the current work, achieved 41.18 U.mg^{-1} , correlating to an increase of over 300 fold in the specific activity. The highest activity retained for immobilised GDH in this study was 42.3%, the highest reported value for activity retention for covalently immobilised GDH is 1.15% (Baron *et al.*, 1997) an improvement of 37-fold. The loss in activity may be a result of the lysine residue, Lys-201, being involved in the immobilisation of the protein. This amino acid has been identified as essential for enzymatic activity and/or ligand binding in GDH (Jany *et al.*, 1984). Loading the particles to maximal capacity may affect retention of activity as steric hindrance around the active site may occur, thereby, affecting conformational adaptation of the enzyme to the substrate (Knezevic *et al.*, 2006; Li *et al.*, 2007).

Considerable thermal and pH stability was displayed by the various immobilised GDH preparations under all conditions tested, with Prep B-GDH displaying the most increased stability over alternate preparations. Even at the highest temperature tested, 65°C , Prep B-GDH displayed a 29-fold half-life improvement compared to the free enzyme. The increase in activity and enhanced stability at acidic pH's renders immobilised GDH more opportunities for applications in processes requiring these acid conditions (Ramachandran *et al.*, 2006). Improved stability for immobilised enzymes may result from "rigidification" of the enzymes' 3-dimensional structure (Pedroche *et al.*, 2007) through multipoint multi-subunit covalent attachments leading to conformational change resistance induced by the surrounding environment (Mateo *et al.*, 2007, Bolivar *et al.*, 2009). Stability in immobilised enzymes may also be enhanced by the micro-environment provided by the support (Mateo *et al.*, 2007; Pedroche *et al.*, 2007). The structure of the particles, Fig. 3.5, allows proteins to be immobilised in the adjacent strands shielding it from harsh external environments (Jordaan *et al.*, 2009a).



Figure 3.5: Schematic diagram depicting how proteins are immobilised within and around the ReSyn™ particles.

3.6 Conclusions

GDH was successfully immobilised onto ReSyn™ particles. The binding capacity of GDH did not follow the general trend witnessed previously for BSA (Chapter 2) with Prep C binding more protein than Prep B. This may have occurred from an insufficient crosslinking time for the protein to interact with the polymer matrix (time based on BSA binding). This phenomenon will be evaluated in future work. The use of ReSyn™ for the immobilisation of GDH is superior to previously published results indicating the efficiency of the polymer support for enzyme immobilisation.

The immobilised GDH exhibited a 42% activity maintenance after immobilisation. Notably, immobilisation resulted in a broadened pH profile and conferred thermal and pH stability with Prep B having the best stabilisation properties. These results can be potentially useful in the applicability of immobilised GDH for the construction of glucose sensors and NADH co-factor recycling systems.

The successful immobilisation of this enzyme allows for its utilisation in the construction of the overall proposed enzymatic co-factor recycling system.

Chapter 4

Optimisation and Characterisation of ReSyn™ immobilised NADH oxidase

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4.1 Introduction

The need to recycle co-factors in order to realise the large-scale and continuous applicability of oxidoreductases has been outlined in Chapter 1 and 3. The recycling of NAD^+ to NADH is possible through the use of NADH oxidase (NOD; E.C 1.6.3.1) since this enzyme oxidises NADH with concomitant reduction of oxygen (Reed *et al.*, 2001; Riebel *et al.*, 2002). Several forms of this enzyme are available; some lead to the bivalent reduction of the oxygen to peroxide (Niimura *et al.*, 2000; Nishiyama *et al.*, 2001), others result in the tetravalent reduction of the oxygen to water (Reed *et al.*, 2001; Riebel *et al.*, 2002). And others (mammalian) are known to be involved in the univalent transfer of electrons to oxygen to form superoxides (Sarker *et al.* 2001, Reed *et al.*, 2001). The various reaction products can potentially influence the source of the enzyme for realising an intended application e.g. NOD's that produce oxygen reactive species may not be favourable for food based application; in such a case the water-producing enzyme would be preferred (Riebel *et al.*, 2002).

The potential application and evaluation of NOD as a co-factor recycling agent has been reported (Riebel *et al.*, 2002, Geueke *et al.*, 2003). Immobilisation of this enzyme has been reported for application to an electrode biosensor for the measurement of NADH (Mizutani *et al.*, 2000) and membrane biosensor (Compagnone *et al.*, 1995). The improvements provided by covalent immobilisation could help realise the applications of this enzyme. These may include improved stability and higher catalytic activity maintenance, thus benefiting biocatalytic co-factor recycling applications. Sanjust *et al.* (1997) demonstrated the immobilisation of this enzyme onto covalent supports for this purpose.

This chapter focuses on the immobilisation of NOD on ReSyn™ particle preparations with subsequent characterisation of improvements to the physical

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stability of the enzyme. The immobilisation of this enzyme is of importance, both in its own right, in biocatalytic applications, and in demonstrating the self-contained co-factor recycling system in which it will be used as a model enzyme to demonstrate the oxidation of NADH to NAD⁺ with the concomitant reduction of oxygen to water.

4.2 Objectives

- To immobilise NOD on various ReSyn™ preparations and characterise the activity changes under various conditions with comparison to the native free enzyme

4.3 Materials and Methods

4.3.1 Reagents

NADH oxidase from *Bacillus licheniformis* (NOD), β-nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide disodium salt hydrate (FAD), potassium phosphate monobasic (KH₂PO₄) and dibasic (K₂HPO₄) were purchased from Sigma Aldrich.

4.3.2 Immobilisation of NADH oxidase

NOD immobilisation was performed on the various ReSyn™ supports using MilliQ H₂O (pH 8.0). The use of Prep D was discontinued due to the low protein binding capacity (BSA and GDH) and low specific activity of the resulting immobilised enzyme. The use of TEA was further discontinued due to the decreased binding capacity and low specific activity experienced in GDH (Chapter 3). The intention is that the most suitable preparation will be chosen for the co-immobilisation of the enzymes for preparation of a co-factor recycling system (Chapter 5). An aqueous stock suspension of NADH oxidase (0.43 mg.ml⁻¹ adjusted to pH 8.0) was used for immobilisation onto the different preparations. This protein was loaded

according to BSA loading capacities determined in Chapter 2 (2.4.4), including a 10% excess to determine maximum binding capacity of NOD. Covalent immobilisation, recovery and protein quantification was performed as described in Chapter 3 (3.3.3). A standard curve for protein quantification was generated using BSA as the standard protein (see Appendix 2C).

For subsequent experimentation the particles were loaded to maximum capacity according to the preliminary data generated for the binding capacity of NOD.

4.3.3 NADH oxidase assays

The assay for NOD was adapted from the Sigma-Aldrich protocol ([http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General Information/nadh_oxidase.Par.0001.File.dat/nadh_oxidase.pdf](http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General%20Information/nadh_oxidase.Par.0001.File.dat/nadh_oxidase.pdf)) Activity for both free and immobilised NOD was determined by following the kinetic oxidation of NADH to NAD⁺ via spectrophotometric absorbance at 340 nm using a microtiter plate reader (Biotek Instruments, PowerWave HT). The oxidation of NADH to NAD⁺ requires the concomitant conversion of oxygen to hydrogen peroxide/water. The assay reagent consisted of 1 mM NADH and 0.1 mM FAD in 50 mM potassium phosphate buffer, pH 7.0. BSA was shown to be unnecessary in the assay as it did not affect the kinetic activity of the enzyme and was subsequently omitted from the assay reagent as this could potentially interfere with the measurement of activity on the protein immobilisation matrices. One unit of NADH oxidase was defined as the amount of enzyme required to oxidise 1 μ mol of β -NADH per minute at 30 °C. The enzyme reactions comprised 5 μ l of enzyme solution with 195 μ l of reagent. The linear range for the kinetic assay was determined with standard concentrations of NOD. All assays were performed in triplicate and represented as the mean \pm standard deviation. In the control assays, the enzyme solution was replaced with water. With immobilised NOD, the particles with bound enzyme were washed twice with MilliQ H₂O and were made to a total volume of 100 μ l in 20 mM phosphate buffer (pH 7.0). The immobilised enzyme preparations were diluted to within the dynamic range of the enzyme assay.

4.3.3.1 pH profiling

The pH profile assays for both the free and immobilised NOD were performed using the same conditions and substrate concentrations as specified above. However, the phosphate buffer was replaced with the universal buffer pH values of interest and adjusted to the concentrations as specified in 3.3.4 (50 mM phosphate, 50 mM Boric Acid, 33 mM Citric Acid and 50 mM Tris adjusted to pH values between 4-10 using HCl and KOH (Jordaan *et al.*, 2004)).

4.3.4 Thermostability

Thermostability was determined by incubation of the free and immobilised NOD preparations at 40 and 45 °C and the activity was assayed as described in 4.3.3 at 10 min incubation intervals using similar substrate and buffer concentrations (1 mM NADH and 0.1 mM FAD in 50 mM potassium phosphate buffer, pH 7.0).

4.3.5 pH stability

pH stability was determined by incubation of the free and immobilised NOD preparations in universal buffer (specified in 4.3.3.1) ranging from pH 3.0 to 10 at 1 pH unit intervals at 10 min intervals and was assayed using the conditions indicated in 4.3.3.

The half-lives determined from the enzyme deactivation experiments for the thermal and pH stability were assumed to be first order.

4.4 Results

4.4.1 Immobilisation of NADH oxidase

As expected (from previously immobilised proteins on the ReSyn™ matrix) Prep A bound the most enzyme at 1.74 mg.mg⁻¹ followed by prep B and C with 1.29 and

0.84 mg.mg⁻¹ respectively (Table 4.1). These results correlate well with the trend observed for BSA (Chapter 2), albeit with reduced protein load.

Table 4.1: NADH oxidase binding capacity on various ReSyn™ preparations using MilliQ H₂O at pH 8.0.

ReSyn™ Preparation	Binding capacity (mg.mg ⁻¹)
A	1.74 ± 0.004
B	1.29 ± 0.001
C	0.84 ± 0.003

*Raw data sufficient to demonstrate this binding capacity is included in Appendix 2C.

4.4.2 Free and immobilised NADH oxidase assays

The specific activity of an immobilised enzyme is influenced by the nature of the support material and enzyme loading capacity (quantity of enzyme per unit of support material) (Adlercreutz, 1997). The maintenance in activity was relatively low across all samples with Prep A-, B- and C-NOD showing maintenance of 8.6, 15.4 and 6.3% respectively (Table 4.2). In most instances in enzyme immobilisation, the contribution of the weight of the enzyme to the overall specific activity is relatively low, however, in the case of ReSyn™, where the protein weight is in excess over the weight of the support, the contribution of the enzyme to the total weight is comparatively high. Thus, the weight of the immobilised enzyme and the support were taken into account when determining the eventual specific activity of the immobilised enzyme. Prep B-NOD displayed the highest specific activity of 6.75 U.mg⁻¹ which was followed by Prep A-NOD with 5.09 U.mg⁻¹ and Prep C-NOD displayed the lowest specific activity at 1.80 U.mg⁻¹ protein. The activity in relation to the total weight, which involves the protein and support (Units per milligram total weight), was 3.21 and 3.80 and 0.82 U.mg⁻¹ for Prep A- and B- and C-NOD which was lower than that of the specific activity as

the support dilutes the activity of the enzyme (Cao *et al.*, 2003; Mateo *et al.*, 2007).

Table 4.2: Activity displayed by immobilised NOD on the various preparation of ReSyn™.

ReSyn™ Preparation	Maintenance in Activity (%)	Specific Activity (U.mg⁻¹ protein)	NOD Activity (U.mg⁻¹ total)
A	8.6 ± 0.29	5.09	3.21
B	15.4 ± 0.60	6.75	3.80
C	6.3 ± 0.13	1.80	0.82

Fig. 4.1 depicts the lysine residues (highlighted) on the structure of NOD (generated using GenThreader and UCSF Chimera from the protein sequence – GenBank: AAU24067). This was done to give a possible explanation for the low activity maintenance in NOD. The lysine residues which flank the active site may be resulting in the immobilisation of NOD in an unfavoured orientation resulting in the active site not being exposed fully for maximum activity and the orientation of the enzyme may result in substrate diffusion limitations.

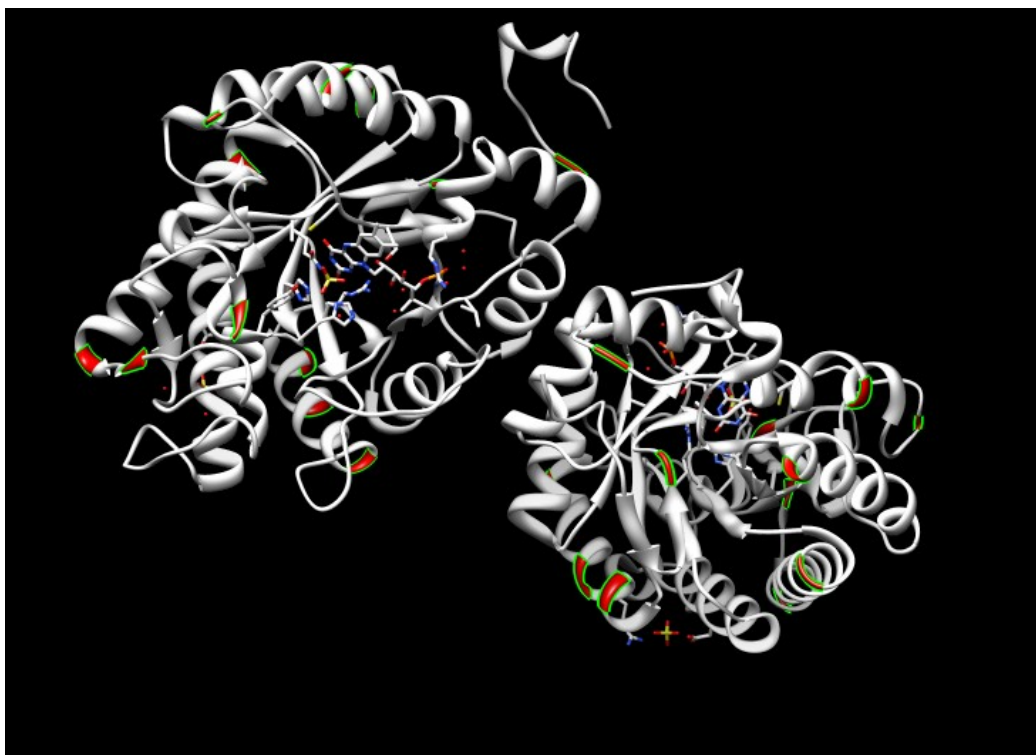


Figure 4.1: Structure of dimeric NOD to indicate the positioning of lysine residues (highlighted in red and green) which may take part in the immobilisation of the enzyme on the polymeric particles. The structure was generated using the protein structure prediction server GenThreader using the *Bacillus licheniformis* NOD protein sequence (GenBank access code: AAU24067). The closely related protein structure (88% homologous) from *Geobacillus kaustophilus* (PDB ID: 3GR7) was visualised using UCSF Chimera. The structures in the active site are the co-factors.

4.4.2.1 pH profiling

The optimal pH for activity for both the free and immobilised NOD preparations was shown to be pH 7.0 (Fig. 4.2). No shift or broadening of the pH profile for immobilised NOD was observed; unlike that seen for GDH which saw broadening of the pH profile in the acidic range (Chapter 3).

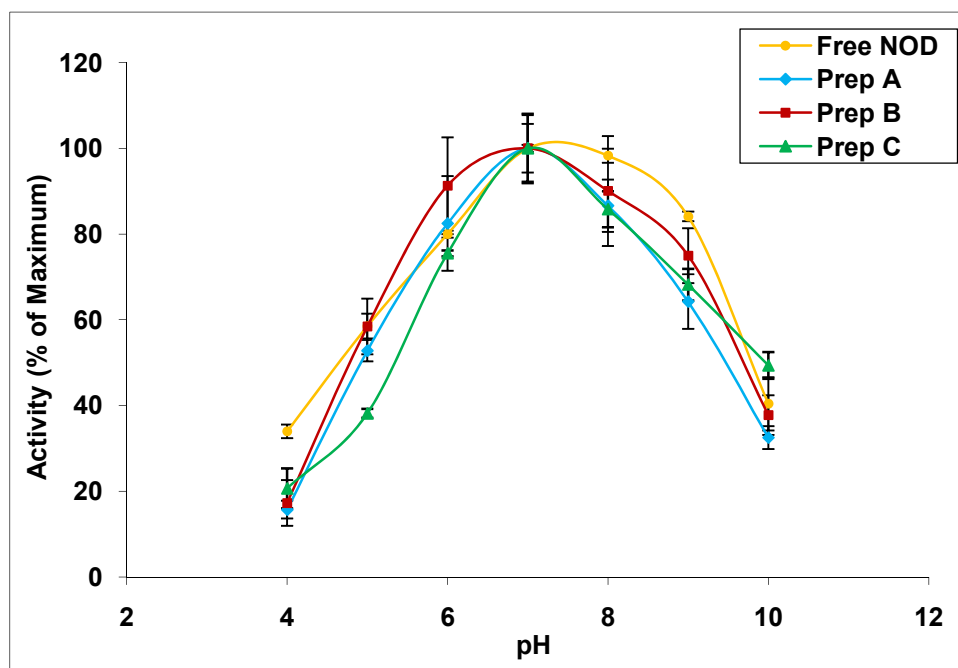


Figure 4.2: pH profile of free and immobilised NOD. The optimal pH of activity was displayed at pH 7.0 for all preparations. The data is presented as the mean of triplicate data \pm the standard deviation.

4.4.3 Thermostability

Thermostability assays were performed at 40 and 45 °C on the free and immobilised enzyme preparations (Fig. 4.3a-b). Prep B-NOD displayed the most improved stability at these temperatures. The half-lives were estimated for the free and immobilised samples and are indicated in Table 4.3. Prep A and B showed improved stability when compared to the free enzyme. The time taken for the free enzyme to reach 50% activity (half-life) was 47 and 40 min respectively, while Prep C-NOD reached 50% activity in 44 and 31 min at 40 and 45 °C respectively and did therefore not appear to confer much improvement in thermal stability.

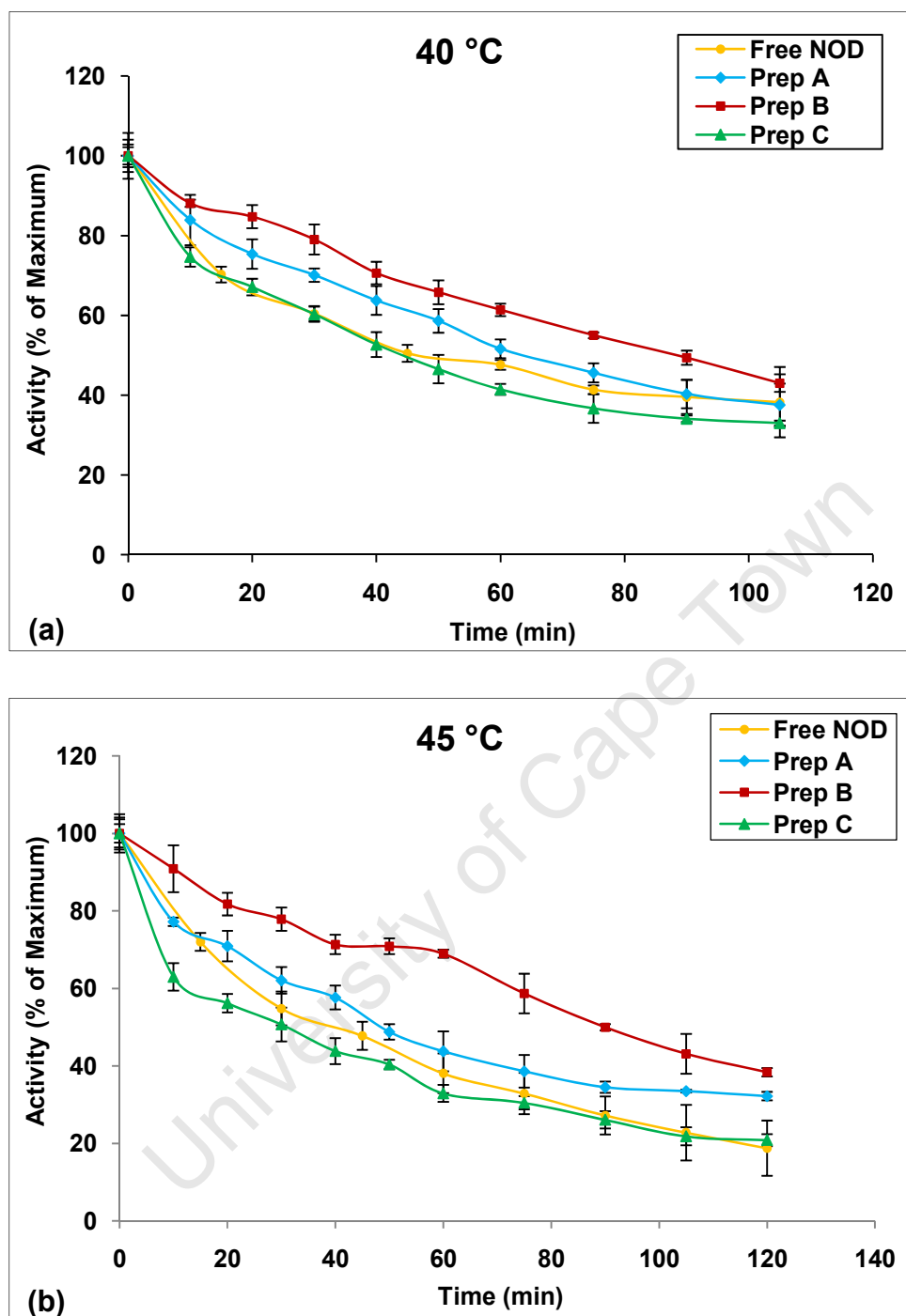


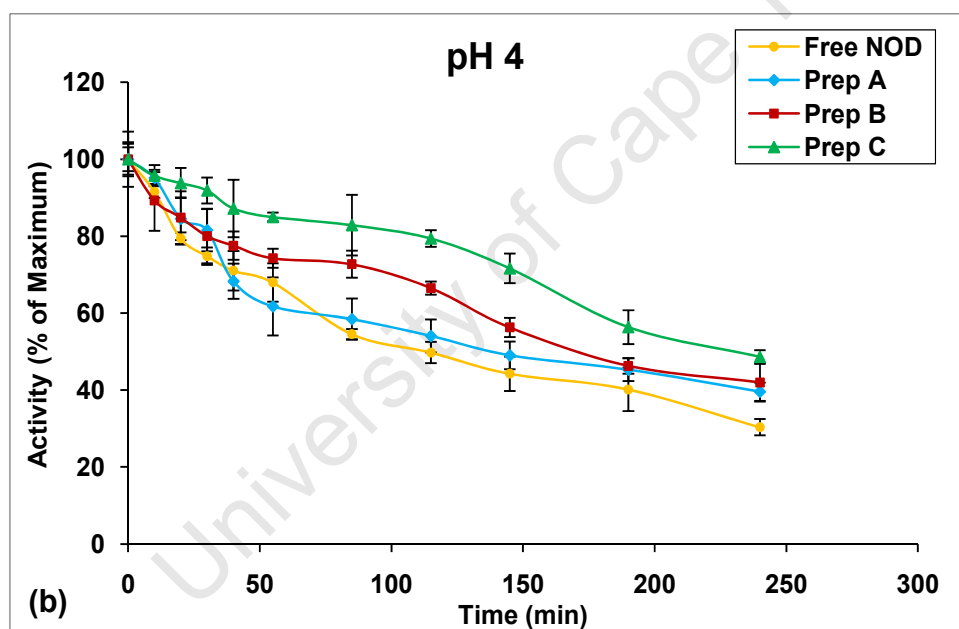
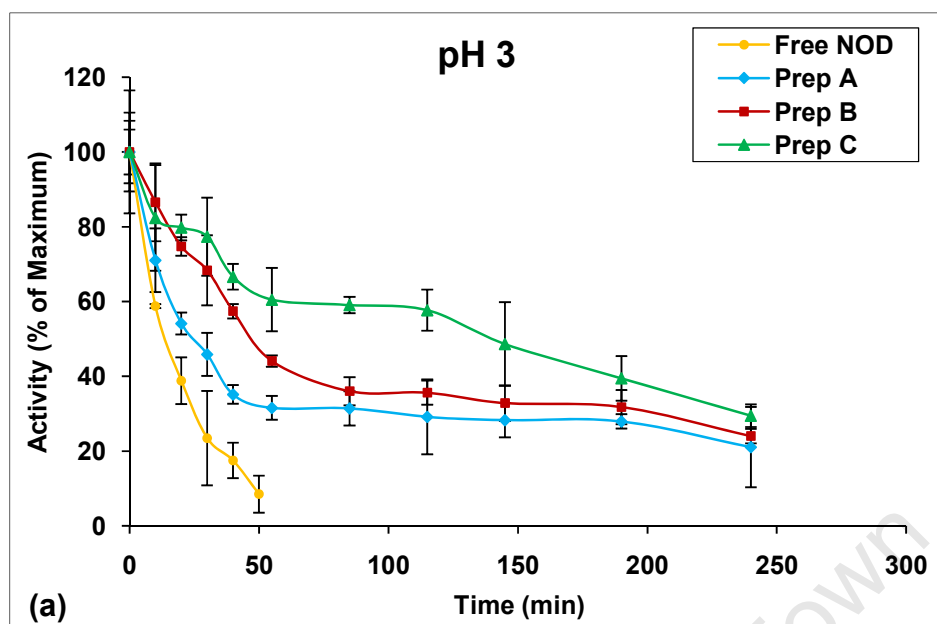
Figure 4.3: Thermal stability at (a) 40, and (b) 45 °C of NOD immobilised on the various particle preparations. The data is presented as the mean of triplicate data \pm the standard deviation.

Table 4.3: Half-life of free and immobilised NOD on the various ReSyn™ preparations at 40 and 45 °C.

Temperature (°C)	Half-life (min)			
	Free NOD	A	B	C
40	47	64	88	44
45	40	49	90	31

4.4.4 pH stability

The half-lives determined for the free and immobilised NOD preparations are summarised in Table 4.4. Prep C-NOD showed the most improved stability at acidic and alkaline pH values of 3.0, 4.0 and 10.0, when compared to alternate preparations. Prep A-NOD showed improved stability in the acidic range, pH 3.0 and 4.0 (Fig. 4.4a-b), whilst at pH 10.0 (Fig. 4.4c) it showed no improved stability over the free enzyme. The most significant pH stability was displayed at pH 3.0, Prep C-NOD reached the half-life at 141 min which translated to a 10-fold improvement and Prep A- and B-NOD displayed 1.8 and 3.4 fold improvements respectively at this pH over the free enzyme.



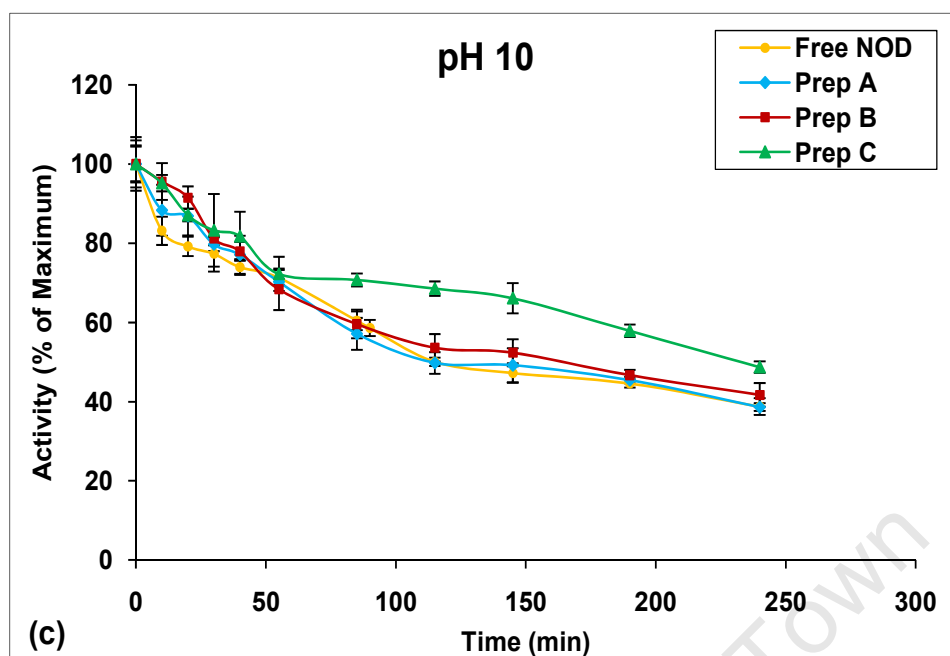


Figure 4.4: pH stability at (a) 3.0, (b) 4.0 and (c) 10.0 of NOD immobilised on the various particle preparations. The data is presented as the mean of triplicate data \pm the standard deviation.

Table 4.4: Half-lives of free and immobilised NOD on the various ReSyn™ preparations at pH 3.0, 4.0 and 10.0

pH	Half -life (min)			
	Free NOD	A	B	C
3	14	25	48	141
4	114	139	173	265
10	116	114	164	233

4.5 Discussion

Immobilisation of NADH oxidase on various preparations of ReSyn™ was performed. These experiments were conducted to evaluate the various preparations for their suitability for immobilisation of NOD with the aim of identifying the most appropriate support for the construction of an eventual co-factor recycling system. The highest binding capacity for this enzyme was achieved with Prep A at 1.74 mg.mg^{-1} . This correlates well with previous experimentation that had showed Prep A having the highest protein binding

capacity (Chapter 2 and 3). But the binding capacity was relatively lower than that of BSA determined in Chapter 2. NOD has a molecular weight of between 50-54 kDa (Higuchi *et al.*, 1993; Gueke *et al.*, 2003) and the binding capacity was expected to be similar, if not higher, than that of BSA, which has a molecular weight of 66.4 kDa. This reduced binding capacity may be due to relatively low lysine content in the enzyme (Hecht *et al.*, 1995), lysine residues are important when immobilising proteins on the ReSyn™ support (Jordaan *et al.*, 2009a; Chapter 2), this may result in the immobilisation rate of the protein to the support being slower (López-Gallego *et al.*, 2005b). The enzyme was not put through the de-salting process using the PD-10 columns as with GDH (Chapter 3), which could further have resulted in a reduced binding capacity due to the presence of interfering compounds, which may have competed with the enzyme for binding sites on the support (Cao, 2005a). The purification using the PD-10 columns was not carried due to the low quantities of the enzyme, thus avoiding loss of enzyme during recovery as experienced with GDH (Chapter 3). Prep B and C immobilised 1.29 and 0.84 mg.mg⁻¹ of NOD respectively, which was also less when compared to the binding capacity achieved when using BSA.

Sanjust *et al.* (1997) covalently immobilised NOD on various supports and the highest binding capacity was obtained when using cross-linked PVA beads functionalised with cyanogen bromide (CNBr) and Sephacryl S-200 HR treated with 2,4,6-Trichloro-1,3,5-triazine (TCT). The supports both achieved a binding capacity of 0.017 mg.mg⁻¹, and immobilisation on ReSyn™ Prep A, B and C displaying a fold improvement of 102, 76 and 46 respectively in the binding capacity. NOD has also been immobilised on single-walled carbon nanotubes (SWCNTs) with a loading capacity of 0.47 mg enzyme.mg⁻¹ SWCNTs (Wang *et al.*, 2010). The high loading capacity of the ReSyn™ particles is probably owing to the large surface area provided by the high amine density and the open fibrous network (Brady & Jordaan, 2009; Jordaan *et al.*, 2009a).

The highest specific activity was achieved with Prep B-NOD, 6.75 U.mg⁻¹ protein followed by Prep A-NOD with 5.09 U.mg⁻¹ protein and Prep C-NOD with the lowest specific activity of 1.80 U.mg⁻¹ protein. NOD immobilised on the cross-linked PVA (CNBr) and Sephacryl S-200 HR (TCT) displayed relatively similar

specific activity of 4.3 and 4.9 U. mg⁻¹ protein respectively (Sanjust *et al.*, 1997). The covalent immobilisation of NOD on glutaryl aminopropyl-PVA (N-hydroxy-succinimide) in the same study achieved a specific activity of 8.3 U.mg⁻¹ protein which was 1.2-fold better compared to the highest specific activity achieved with Prep B-NOD. The low activity displayed by immobilised NOD may be a result of enzyme deactivation due to the interaction of the enzyme and the support (Adlercreutz, 1997; Cao *et al.*, 2003), or it may be due to the steric hindrance which may result from high-enzyme loading (Adlercreutz, 1997; Knezevic *et al.*, 2006; Li *et al.*, 2007) and the orientation of the enzyme following immobilisation (Cecchini *et al.*, 2007; Kim *et al.*, 2008) leading to potential mass-transfer limitations with regards to the substrate entering, and products exiting the internal spaces of the particle (Adlercreutz, 1997; Knezevic *et al.*, 2006; Li *et al.*, 2007; Cecchini *et al.*, 2007; Kim *et al.*, 2008). The stabilising effect of PEI has previously been shown to play a role in the stabilisation of oxygen-sensitive nitrilases (Mateo *et al.*, 2006b). Mateo *et al.* (2006b) attributed this to the reduced solubility of oxygen in the polymer. Since NOD utilises oxygen as a substrate, the reduced oxygen solubility may have directly reduced the enzyme activity and may be a contributing factor in the low retention of activity displayed by PEI immobilised NOD.

There was no observed shift in the pH profile of the immobilised enzyme as both the free and immobilised enzyme had a pH optimum of 7.0. Immobilisation led to improved thermal and pH stability; and this advantage may expand the range of conditions suitable for enzyme function in various applications (Mateo *et al.*, 2007; Wang *et al.*, 2010). Increased thermostability was achieved with NOD immobilised on Prep A- and B at the temperatures 40 and 45 °C while Prep C-NOD did not display improved stability. The most improved stability was displayed by Prep B-NOD, similarly to GDH in Chapter 3, with a fold improvement of 1.9 and 2.3 at 40 and 45 °C respectively compared to that of the free enzyme. The pH stability displayed a different trend, with Prep C-NOD displaying the highest improved stability over alternate preparations. The preparation showed 10.0, 2.3 and 2.0 fold improvements for pH 3.0, 4.0 and 10.0 respectively when compared to the non-immobilised enzyme. Prep B-NOD displayed its highest pH stability at the acidic pH of 3.0 with a fold improvement of 3.4 over the free enzyme. Only

moderate improvements in stability were previously noted for NOD (Sanjust *et al.*, 1997) and this correlates with the results obtained from this study. Even in this case the degree of crosslinking of the particles may affect the stability conferred. This improved stability for the immobilised preparations of this enzyme may be a result of the reasons mentioned in Chapter 3 (3.5) based on the structural changes in the enzyme post immobilisation. Prep A, the most loosely linked polymer compared to alternate preparations, showed little or no improved stability at the pHs and temperatures tested. Prep B seems to provide a better micro-environment in elevated temperatures while Prep C in acidic pH conditions. In the case of GDH (Chapter 3), Prep B resulted in the most improved temperature and pH stability.

4.6 Conclusions

Immobilisation of NOD on ReSyn™ particles was successfully achieved. The binding capacity of NOD followed the general trend observed for BSA (Chapter 2). The binding capacity for the immobilisation of NOD using ReSyn™ is superior to previously published results.

The enzyme activity maintenance achieved for NOD was 15.4%, which is considerably lower than that achieved for GDH at 42%. This may have been due to the reduced solubility of oxygen in PEI (Mateo *et al.*, 2006). Post modification methods to alter the charge of particles are currently being evaluated to improve enzyme activity. Prep B conferred superior thermal and pH; Prep C conferred a high degree of pH stability. The stability conferred by the immobilisation could potentially expand the application of NOD to NAD⁺ co-factor recycling in immobilised enzyme systems.

The successful immobilisation of NOD indicates its suitability for inclusion into the co-factor recycling system.

Chapter 5

Construction of an NADH cofactor recycling system using an enzyme-coupled system

5.1 Introduction

Various methodologies to recycle expensive co-factors have been developed (Chapter 1; Section 1.4) and the enzymatic-based co-factor recycling method is preferred due to its selectivity and efficiency (Wichmann & Vasic-Rački, 2005; Liu & Wang, 2007). The recycling of co-factors is deemed necessary for realising the industrial applicability of enzymes belonging to the class oxidoreductases as they require the addition of stoichiometric amounts of these co-factors, which is not desirable for industrial applications due to cost (Wichmann & Vasic-Rački, 2005; Liu & Wang, 2007; Kohlmann, *et al.*, 2008). For successful recycling the reduced and oxidised form of the co-factor must effectively shuttle between the respective enzymes resulting in a continuous system (Liu *et al.*, 2009).

The preparation of self-contained enzymatic co-factor recycling systems, i.e. containing enzymes and co-factors, have been explored using capsules, membrane reactors and solid-support attachment (Wichmann & Vasic-Rački, 2005). In microcapsules and membrane reactor systems the major challenge is to retain the co-factors and enzymes since they are not bound to any surface in these systems, which may result in leaching of these small molecules (Liu *et al.*, 2009). Diffusional constraints, particularly with regards to the permeability of substrates and products have to be considered (Cao, 2005a). Low permeability can result in low conversions through substrate limitation or product inhibition, reducing the total turnover number of the co-factor, defined as the μmol product formed per μmol co-factor in the reaction (Liu *et al.*, 2009). The enlargement of co-factors and their subsequent immobilisation with enzymes is a method which has been applied to alleviate this problem in constructing co-factor recycling systems (El-Zahab *et al.*, 2004). The immobilised systems allow ease of recovery and re-use especially when expensive enzymes are involved (Mateo *et al.*, 2007).

The display of co-factor recycling ability by both GDH and NOD is of scientific interest as these two enzymes are important biocatalysts (Riebel *et al.*, 2002; Geueke *et al.*, 2003; Baron *et al.*, 1997; Baik *et al.*, 2003). A self-contained co-factor recycling system has a number of potential applications spanning from production of chiral compounds by co-factor dependent dehydrogenases (Hummel & Kula, 1989; Hummel, 1999) to biomedical applications such as in the field of biosensors, diagnostics (Silber *et al.*, 1996; Costa *et al.*, 2004; Serban & El Murr, 2006; Rahman *et al.*, 2009), and therapeutics (Kotorman *et al.*, 1994; Costa *et al.*, 2004).

This chapter demonstrates proof-of-principle for the construction of a co-factor recycling system comprised of immobilised GDH-NOD with encapsulated pegylated NADH co-factor.

5.1.1 Selection of polymer support for cofactor recycling system preparation

Of importance when considering a suitable support for the co-factor recycling system is the specific activity, which is defined as the units of enzyme activity per total weight. This is indicative of the efficiency of the enzyme to carry out the desired reaction. The support dilutes the activity of the enzyme and therefore the specific activity of the support is an important variable for the choice of the support (Sheldon *et al.*, 2005; Cao *et al.*, 2003; Cao, 2005a,b). Stability conferred to the enzyme is further of importance for its potential applications as they are most likely to be applied outside of their native conditions (Iyer & Ananthanarayan, 2008). Several industrial processes require unnatural pH and temperature conditions, since these may lead to higher process production rates (Iyer & Ananthanarayan, 2008). For therapeutic and biosensor applications, the pH and temperature of the environment are further important e.g. the internal environment of the body (Gupta *et al.*, 2002).

The results for the various preparations are outlined in Table 5.1 in the form of scores highlighting performance standards; where the preparation which performed best was given the highest score of 3 while the poorest performance was awarded a score of 1. Prep A-GDH displayed the highest specific activity, 41.18 U.mg^{-1} , followed by Prep B- and C-GDH with 33.22 and 33.01 U.mg^{-1} respectively. Immobilisation of NOD yielded comparatively lower specific activities due to lower enzyme activity maintenance after immobilisation. NOD immobilised on Prep B displayed the highest specific activity of 6.75 U.mg^{-1} this essential as NOD is the rate limiting reaction when comparing the specific activity of the enzymes following immobilisation. For both enzymes, preparation B displayed the highest maintenance in activity; this affects cost of constructing the system as it will determine the quantity of enzyme units to be incorporated in the system to achieve the desired reaction rate.

In general preparation B provided the most pronounced improvement in pH and thermal stability (Table 5.1). For the thermostability experiments conducted, GDH and NOD showed vast improvement when they were immobilised on Prep B compared to the alternate preparations. Furthermore, the pH stability of GDH was most improved when immobilised on Prep B. However, the pH stability of NOD was the best with Prep C, with Prep B not being far off (Table 5.1). Preparation A showed the lowest stability improvement for both enzymes under all the conditions tested. In overall, Preparation C had the lowest score of 16 while Prep A was only higher by 2 points.

The results indicate that Prep B was the most suitable matrix for the preparation of the co-factor recycling system as is indicative by the total score of 25.

Table 5.1: Scores allocated for results obtained from the immobilisation of GDH and NOD on ReSyn™ preparation A, B and C.

Parameters	GDH			NOD		
	A	B	C	A	B	C
Binding Capacity	3	1	2	3	2	1
Enzyme Activity Maintenance	1	3	2	2	3	1
Specific Activity	3	2	1	2	3	1
Temperature stability	1	3	2	1	3	2
pH stability	1	3	1	1	2	3
Total	9	12	8	9	13	8

A, B, and C indicate the different ReSyn™ Preparations. Scores were assigned for the results obtained from the immobilisation GDH and NOD (Chapter 3 and 4 respectively). The scores indicate: 3 – highest; 2 – intermediate; 1 – lowest result. The colours indicate level of performance as follows: ■ - best, ■ - average and ■ - worst.

5.2 Objectives

- To co-immobilise equi-amounts of GDH and NOD on the chosen ReSyn™ Prep B particles and achieve corresponding forward and reverse reaction rates to ensure recycling efficiency
- Entrapment of polyethylene glycol enlarged-NADH with the co-immobilised enzymes to construct final system
- Achieve proof-of-concept by demonstrating co-factors recycling in the self-contained system

5.3 Material and Methods

5.3.1 Reagents

Polythyleneglycol (M_r 20 000) modified-NADH (PEG-NADH) was obtained from Julich Fine chemicals, ethanolamine, NADH and NOD, D(+)-glucose, FAD, KH_2PO_4 , K_2HPO_4 , were obtained from Sigma; NAD^+ was obtained from Roche and GDH was obtained from Codexis.

5.3.2 Construction of the cofactor recycling system

5.3.2.1 Co-immobilisation of GDH and NOD

Prep B particles were prepared as described in Chapter 2. The amount of enzyme and particles used in the co-immobilisation were determined as shown in Appendix 3A. In brief: the co-immobilised units of GDH and NOD were determined using the results of specific activities of the immobilised enzymes; this was done to obtain an ideal stoichiometric conversion rate; i.e. each enzyme would act on the relevant co-factor for a continuous reaction. The enzyme loading capacity of the particles was reduced for the purposes of immobilisation (20%) to allow complete binding of both enzymes. The immobilisation, washing and recovery processes were done as previously described (Chapter 2, 3 and 4). Following the last washing and recovery step, the particles with bound protein had 100 μl of MilliQ H_2O added and were assayed for individual NOD and GDH activity following mixing to homogeneity using a vortex (described below).

5.3.2.2 Co-factor entrapment

Following co-immobilisation of the enzymes on the particles; the cofactor was entrapped within one of the suspensions by mixing 100 μl of PEG_{20000} -NADH and incubated at room temperature with gentle agitation for 30 min (10.1 μM). The mixture was centrifuged for 5 min at 6000xg to recover the unbound PEG-NADH for quantification of the entrapped co-factor. The recovered solution of PEG-NADH was quantified by spectrophotometric absorbance at 340 nm. Entrapment of the

PEG-NADH was effected as described by Jordaan *et al.*, 2009a. The particles were lyophilised overnight at a shelf temperature of 0 °C and vacuum of 0.3 mBar (FreeZone[®] Triad[™] Freeze Dry System, Labconco Corporation), lyophilisation brings the unreacted groups into close proximity resulting in further linkages or bonds formed allowing the co-factor to be enclosed in the system but still be able to shuttle between enzymes during the reaction (Jordaan *et al.*, 2009a). The lyophilised product was washed twice with 500 µl of MilliQ water and recovered through centrifugation (6000xg). The particles were then re-suspended with 100 µl of 20 mM phosphate buffer pH 7.5. To quench excess aldehyde functionality of the particles 100 µl of 20 mM ethanolamine was added to the particles and incubated for 30 min at room temperature with constant gentle agitation. The particles were washed 5 times with 500 µl phosphate buffer (50 mM pH 7.5) and were re-suspended in 100 µl of 20 mM of the same buffer (pH 7.5). The suspension without co-factor was subjected to the same lyophilisation procedure to determine the loss in enzyme activity due to this step.

5.3.3 GDH and NOD enzyme activity assays

The optimal pH for assay was chosen as the intersection in pH profiles of immobilised enzyme preparations to ensure the optimal catalytic rate for both enzymes (Chapter 2 and 3). Fig. 5.1 shows the pH profiles of the immobilised enzymes with the intersection at pH 7.5 being the choice to conduct the assays.

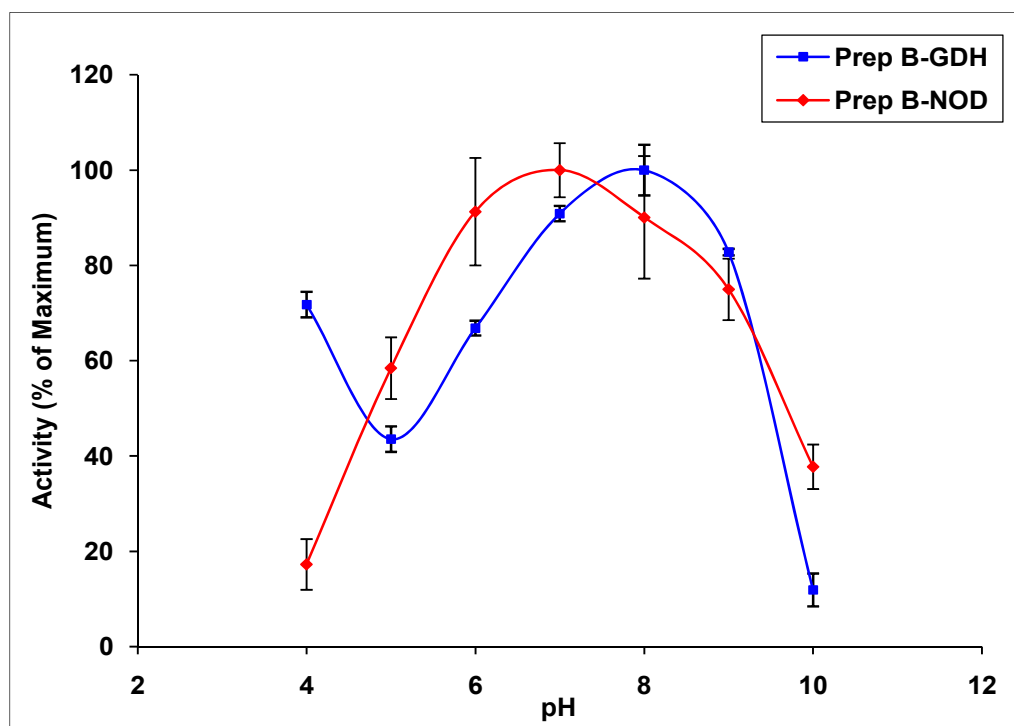


Figure 5.1: pH profile of immobilised NOD and GDH on Prep B ReSyn™ particles.

Prior to co-factor entrapment the activity of the co-immobilised enzymes was performed, and the activity of each enzyme was assayed individually. For GDH the appearance of free NADH was monitored spectrophotometrically under the conditions described in Chapter 3; and for NOD activity the disappearance of NADH was monitored as described in Chapter 4. This was done to determine the similarity in rates for the 2 enzymes and to validate the stoichiometric calculations for enzyme activity. The assays were performed in triplicate.

The NADH oxidase half-reaction, which involves the oxidation of NADH, was conducted by measuring the oxygen consumption rate in a closed vessel. The assay reagent consisted of 50 mM D (+) glucose, 0.01 mM FADH, 50 mM phosphate buffer pH 7.5 at 30 °C. The reaction rate was measured in a closed system using a Jenway-970 oxygen meter for 120 min with continuous magnetic stirring at 20 rpm. The oxygen probe was calibrated according the manufacturer's protocol before use. The reaction comprised of 25 µl of the recycling suspension in a 20 ml assay volume. The co-immobilised enzyme preparation without the

inclusion of PEG-NADH was used in the control reaction. No oxygen consumption was noted for this control experiment. The re-usability of the system was evaluated by recovering and re-assaying of the particle suspension. The particles were recovered from the assay reagent by centrifugation at 3901xg for 10 min at 10°C using a swing-out bucket rotor. The particles were re-suspended with 25 µl of phosphate buffer (20 mM pH 7.5) and the reaction was initiated by the addition of the particles to fresh reaction medium.

The GDH enzyme half-reaction, which involves the reduction of NAD⁺, was monitored using a chiral assay developed in our laboratory to follow the consumption of the substrate D (+) glucose. The chiral signature of glucose was determined by running a circular dichroism spectrum on a Chirascan spectropolarimeter/CD spectrophotometer (description of the instrument is outlined in Appendix 3B) (Applied Photophysics, UK). The chiral signature of glucose was determined by running a standard concentration of glucose (100 mM). Standard curves in the signature range (193 – 200 nm) of glucose were generated using concentrations from 10 mM to 60 mM. These standard curves were used to determine the linear range of the assay and to determine the suitability of each wavelength for glucose chiral adsorption (extinction coefficient) (Refer to Appendix 3C). The reaction mixture was analysed in the spectral range to identify any potential interference by any of the assay reagents (Appendix 3D).

From the data analysis a suitable wavelength of 197nm was selected to monitor the reaction since the glucose concentration displayed a linear relationship to the chiral signature and the assay concentration of glucose (50 mM) was within range for the spectropolarimeter. The reaction was monitored under the same conditions (pH 7.5, 30 °C); the control included the reaction mix with the particle suspension with the co-immobilised enzymes, without the encapsulation of the pegylated co-factor.

5.4 Results

5.4.1 Activity of co-immobilised GDH and NADH

The co-immobilised enzymes were assayed for activity to determine the rate of the 2 half-reactions. The average rate \pm standard deviation for the GDH half-reaction was $190.88 \text{ mOD} \cdot \text{min}^{-1} \pm 4.56$, and for the half-reaction of NOD it was $215.64 \text{ mOD} \cdot \text{min}^{-1} \pm 5.23$. These reaction rates correlate well for stoichiometric conversion of the co-factor and validate the calculations for equivalent conversion.

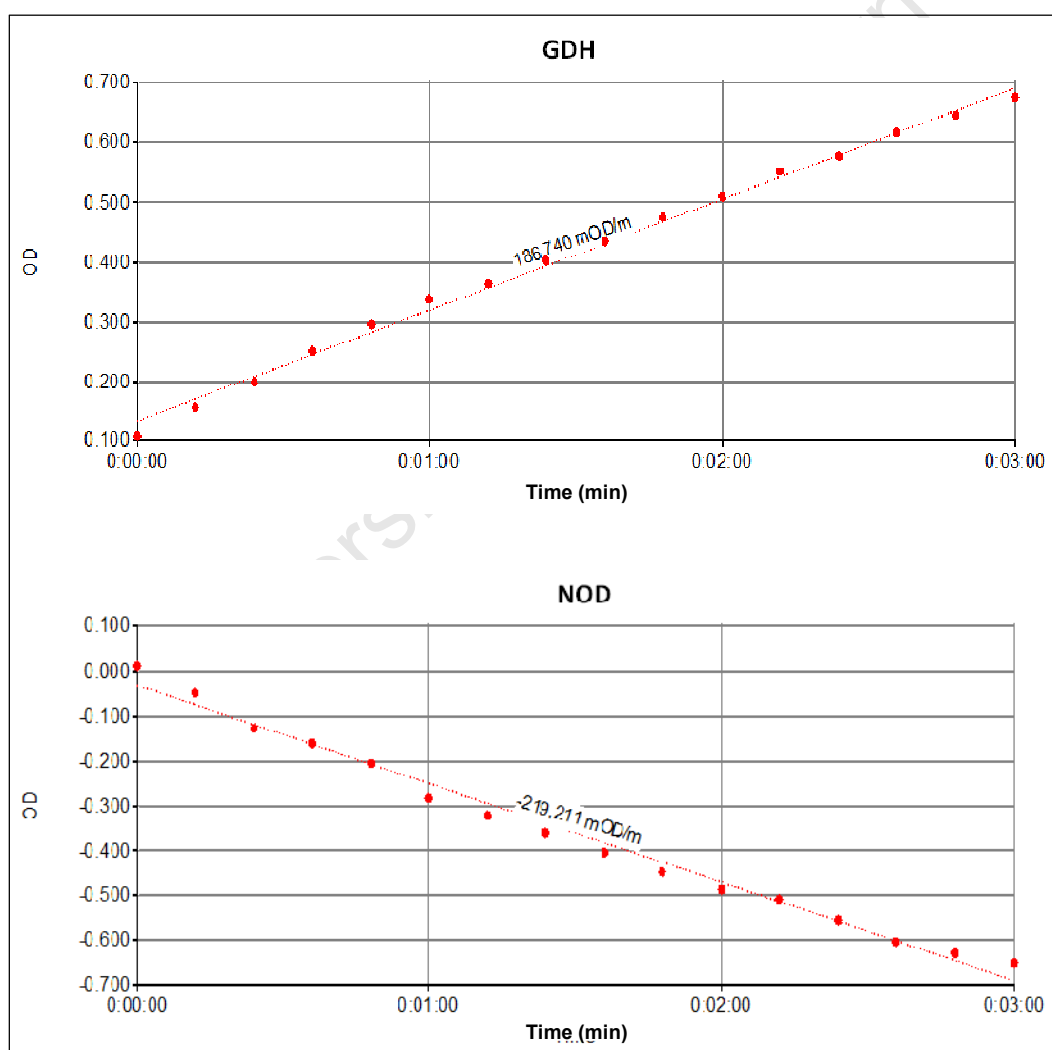


Figure 5.2: The reaction rates obtained with co-immobilised GDH and NOD assayed separately with free co-factor.

The particles with the enzymes were lyophilised for the entrapment of the co-factor and this resulted in the loss of enzyme activity with NOD and GDH maintaining 84% and 88% respectively. Lyophilisation results in the loss of surface water residues decreasing the polarity of the enzyme's micro-environment leading to a change in conformational mobility (Lee & Dordick, 2002). This effect on the enzyme may be prevented through the use of a protectant during this process as it may preserve and protect the enzyme (Lee & Dordick, 2002).

5.4.2 GDH-NOD cofactor recycling system assay

The oxygen probe was calibrated in water at 30 °C. The corresponding oxygen concentration of this solution was 7.56 mg.L⁻¹. This was set as 100% saturation for the probe. Relative concentrations of oxygen from the experiment were converted to corresponding concentrations. The molarity of the oxygen was subsequently determined and is plotted in Fig. 5.3. The initial oxygen content peaked at 4.58 µmol. The total oxygen consumption was achieved for the system. Since only 0.0288 µmol of co-factor was incorporated with the particles, the co-factor regeneration was calculated at 142 times during the experiment. The recycling assay, indicated a further 136 regenerations. The reaction rate determined between the time points 10 – 50 min decreased from 0.0478 µmol.min⁻¹ to 0.0367 µmol.min⁻¹, a 23% reduction in the activity. This half-reaction had a specific activity of 0.619 U.mg⁻¹ particles. The control indicated that no reaction occurred without the co-factor.

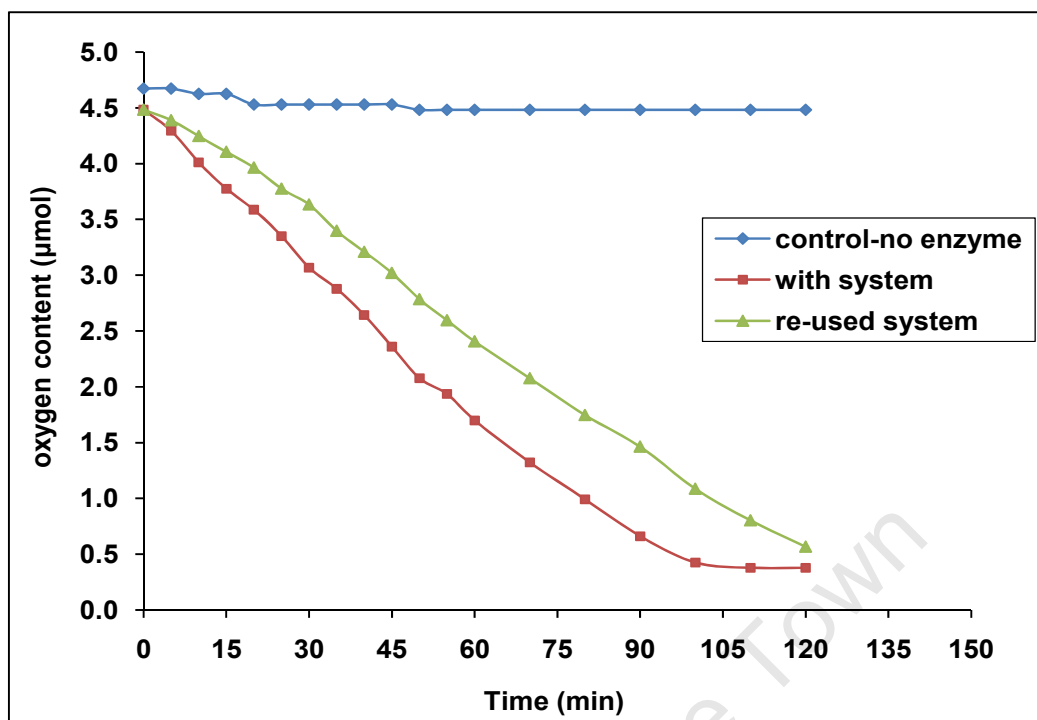


Figure 5.3: Graph depicting the depletion of dissolved oxygen by the GDH-NOD coupled co-factor recycling system.

The consumption of glucose requires the presence of NAD^+ , which indicates the successful shuttling of the co-factor between the two enzymes. The amount of glucose consumed was determined using the standard curve at 197 nm (see Appendix 3C). The negative control for this experiment indicated the particles absorbed glucose. However, after the initial adsorption, a measurable rate for glucose consumption could be achieved. The adsorption of glucose appeared to stop after 72 sec of incubation.

The rate for this half-reaction was determined after glucose adsorption (Fig. 5.4). The rate of reaction determined between the times 324 - 414 sec was $0.309 \mu\text{mol} \cdot \text{min}^{-1}$, equating to a specific activity of $10.18 \text{ U} \cdot \text{mg}^{-1}$ particles.

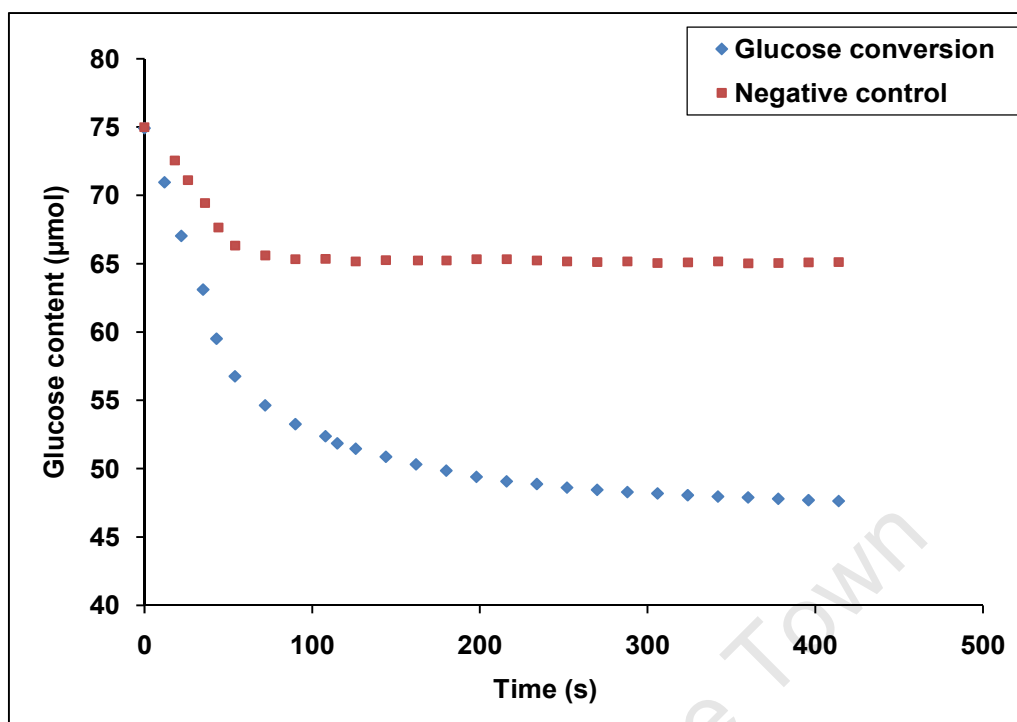


Figure 5.4: Consumption of glucose by GDH and absorption of glucose by the polymer particles.

From the interesting observation that glucose was adsorbed by the particles an assay was done to determine whether NADH could be adsorbed to the particles and thereby potentially negate the requirement for pegylated NADH. To evaluate this, the particles containing co-immobilised enzymes were incubated with 1 mM non-pegylated NADH for an hour at room temperature with constant agitation. This suspension was centrifuged and the supernatant was quantified for unbound NADH by spectrophotometric absorbance at 340 nm. The particles appeared to absorb 0.975 ± 0.004 mM NADH from the 1 mM solution. To determine whether the absorbed co-factor could be utilised by the immobilised enzyme, possibly resulting in the elimination of utilising PEG-NADH, an oxygen consumption assay described in 5.3.3 was performed using the particles containing the co-immobilised enzymes and the absorbed NADH co-factor. No oxygen depletion was detected using the assay, indicating the necessity to encapsulate the enlarged co-factor. The NADH may have diffused away from the particles in the dilution solution, displaced by the reaction ingredients, or was somehow not available for shuttling between the enzymes for recycling, e.g. chelated to the polymeric material.

5.5 Discussion

The results from the individual immobilisation of the enzymes formed the basis for selecting the support used, Prep B (Section 5.1.1), for the construction of the co-factor recycling system. The recycling reaction was demonstrated using assays for each of the systems half-reactions (Fig. 5.5). The entrapment of the co-factor was required for activity of the system as demonstrated by Jordaan *et al.* 2009a. The oxidative reaction displayed an NADH recycle of 142 times over a period of two hours. The system showed a 77% activity maintenance after recovery and re-use. The activity lost could have resulted from poor particle recovery (25 μ l recovered from 20 ml reaction), or perhaps due to enzyme deactivation which may have occurred during the recovery and recycling process (Wang *et al.*, 2010). The reduction reaction displayed a total of NADH recycle of 98.61 times in one hour, similar to that of the oxidative reaction.

The reactions taking place in the system were monitored using glucose and oxygen consumption assays to verify that the system was working in both directions, and to confirm that co-factor recycling was occurring. These assays did not correlate well with each other where the reaction rate of the oxidative reaction carried out by GDH was higher than that of the reduction reaction of NOD. The NOD reaction relied on the consumption of oxygen in the reaction mix and the presence of a head space in the reaction setup may have resulted in the dissolution of oxygen into the reaction mix, thereby, replaced used oxygen through diffusion. Although these results did not correlate well with each other, the aim was to demonstrate that the recycling reaction was indeed taking place. From the quantitative results obtained the system clearly demonstrated that the system was capable of NADH/NAD⁺ recycling.

Various systems have been constructed to recycle co-factors in immobilised systems, but there are limited examples of where enzymes and co-factors are immobilised on the same support (Liu *et al.*, 2009). El-Zahab *et al.* (2004) immobilised GDH, lactate dehydrogenase and elongated NADH using various

spacers on porous silica particles and the highest specific activity achieved was 4.10 U.mg^{-1} when the longest spacer was used translating to 2.48 fold improvement in the rate of the system in this study. The work of Liu *et al.* (2009) immobilised NAD(H), glutamate and lactate dehydrogenase separately to silica nanoparticles and the highest specific activity achieved was 0.0418 U.mg^{-1} . The ReSyn™ co-factor recycling system displayed a 243-fold improvement with a specific activity of 10.2 U.mg^{-1} (support). This may be due to that the system constructed by Liu *et al.* (2009) largely depended on particle mobility and collision for co-factor recycling to occur and for the reaction to be continuous, since each component was immobilised on its own set of silica particles. This research demonstrates that this is not required in self-contained systems.

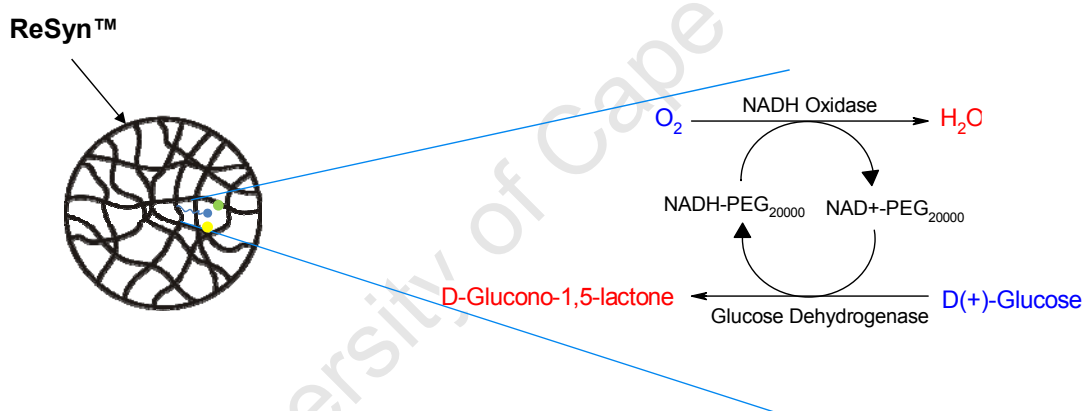


Figure 5.5: Diagram depicting the constructed co-factor recycling systems using GDH and NOD. The system is designed so that each polymer particle consists of the enzymes with entrapped co-factors acting as an independent recycling system.

Various applications which use NAD^+ - and NADH-dependent oxidoreductases require the recycling of NAD^+ and NADH respectively (Geueke *et al.*, 2003). The two enzymes NADH oxidase and glucose dehydrogenase provide useful means for the recycling of NAD^+ and NADH for co-factor dependent systems (Riebel *et al.*, 2002; Hummel, 1999).

5.6 Conclusions

ReSyn™ preparation B appeared to be the most suitable support for the construction of the co-factor recycling system due to the improved properties displayed by the individual immobilisation of GDH and NOD with comparatively high enzyme activity maintenance. Following lyophilisation the immobilised NOD and GDH maintained 84% and 88% of their respective activities.

The ReSyn™ particles were capable of absorbing glucose and non-PEGylated NADH. The system requires the encapsulation of PEG₂₀₀₀₀-NADH for its functionality. This may limit the application of these systems due to the increased cost of such co-factors.

The construction of a co-factor recycling system consisting of entrapped PEG₂₀₀₀₀-NADH with the co-immobilised enzymes, GDH and NOD, has been successfully demonstrated. The system had a reaction rate of 10.18 U.mg⁻¹.support which was higher than the currently reported values. We have demonstrated that the co-factor is available for shuttling between the two enzymes. The system was re-used successfully and maintained 77% of activity.

Chapter 6

Conclusions and Future Work

Dehydrogenases have found application in various fields including biocatalysis, the development of biosensors and therapeutics (Liu & Wang, 2007). The limitations hampering the industrial applicability of oxidoreductases stem from the requirement for stoichiometric amounts of co-factors, which are often expensive, and are further relatively unstable in the enzymatic reactions. Several methods have been developed to partially overcome this limitation. The enzymatic co-factor recycling method is the most favoured due to its high efficiency, selectivity and specificity. The development of a self-contained enzymatic co-factor recycling system can assist in realising the application of these enzymes.

A support that has a potential high protein binding capacity and enables co-factor entrapment provides the prospect for the development of an immobilised co-factor recycling system. The proprietary technology of ReSyn™ particles displayed superior binding capacity compared to alternate immobilisation technologies and the matrix has previously been shown to be capable of co-factors entrapment as described by Jordaan *et al.*, 2009a.

The immobilisation of enzymes has been shown to enhance properties, such as activity and stability, which are essential in realising the applications of enzymes (Mateo *et al.*, 2007). The immobilisation of glucose dehydrogenase and NADH oxidase was successfully demonstrated. The individual immobilisation of GDH and NOD is of interest as they are both industrially important in the recycling of NADH and NAD⁺ respectively. The immobilisation achieved using ReSyn™, 1.22 and 1.74 U.mg⁻¹ for GDH and NOD respectively, was superior to previously published research (Baron *et al.*, 1997; Wang *et al.*, 2010). Both immobilised enzymes displayed improved thermal and pH stability. In addition, GDH displayed a broader than usual pH profile, and increased activity in the acidic range. These features

may enable or improve applications of the enzyme, e.g. synthesis of gluconic acid in the food industry (Ramachadran *et al.*, 2006). The immobilisation of NOD resulted in relatively low enzyme activity maintenance. This limitation may need to be overcome for realising potential applications such as biocatalysis. This could potentially be achieved through post-modification of the polymer (Jordaan *et al.*, 2009a), or through investigating the use of stabilising agents such as buffers (Tsitsimpikou *et al.* 1995). Another method for improving enzyme immobilisation, and probably therefore, enzyme activity, is to control the orientation of the enzyme on the support during immobilisation. This can be achieved by selectively introducing predetermined amino acid residues, in this case Lys residues, on positions further away from the active site through the use of protein engineering using methods such as site-directed mutagenesis (Persson *et al.*, 1990; Rao *et al.*, 1998; Turkova, 1999; Cecchini *et al.*, 2007; Kim *et al.*, 2008).

The co-factor was successfully entrapped within the matrix containing the co-immobilised proteins. With immobilised enzymes, the co-factor has to be readily available and in close proximity to allow shuttling between the two enzymes (El-Zahab *et al.*, 2010; Liu *et al.*, 2009). The system was shown to be active in the oxidation and reduction of NADH, achieving a 10.18 U.mg^{-1} specific activity and successfully recycling the co-factor 142 times. The system could be recovered and recycled with a 77% efficiency. The potential exists to increase the efficiency of the system by modifying various parameters such as the concentration of substrates and co-factor.

Thus the concept of a co-factor recycling system has been successfully demonstrated. These systems may expand the applications base of fields such as biosynthesis, biosensors, diagnostics and therapeutics (Liu *et al.*, 2009). In its current form, the device is capable of highly efficient oxygen consumption. Oxygen scavenging enzymes have application in for instance fluorescence microscopy (Vogelsang *et al.*, 2008) and food preservation (Meyer & Isaksen, 1995). Enzymatic systems for oxygen reduction have been extensively for food preservation processes such; an example of which is the catalase-glucose

oxidase system for flavour-protection in citrus juices and to prevent lipid oxidation in mayonnaise (Meyer & Isaksen, 1995). Enzymatic oxygen-scavenging systems are further useful in fluorescence spectroscopy; which prevents photobleaching and blinking of fluorescent dyes which can interfere with imaging (Aitken *et al.*, 2008; Vogelsang *et al.*, 2008). This system may also enable the preparation of alternate co-factor recycling systems using different enzymes for evaluation for an application.

This work has successfully proven the concept of developing a functional NADH/NAD⁺ co-factor recycling system using immobilised GDH, NOD with entrapped PEG-NADH on the proprietary particle technology termed ReSyn™. The high protein binding capacity and improved enzyme stability, provided by the particles, can potentially expand applications of dehydrogenases. Enzymatic co-factor recycling systems with stable and continuous activity may result in the development of efficient and improved biosynthetic, biosensor, diagnostic and therapeutic system.

Appendices

Appendix 1A: Data for BSA binding using MilliQ water and TEA pH 8.0

Appendix 2A: Standard curve of GDH in MilliQ water pH 8.0

Appendix 2B: Standard curve of GDH in TEA buffer pH 8.0

Appendix 2C: Standard curve of BSA in MilliQ water pH 8.0

Appendix 3A: Determination of enzyme loading and amount of Prep B particles to required for co-factor recycling construction

Appendix 3B: Assay design for the detection of D(+) glucose using Chirascan CD spectrophotometer

Appendix 3C: Standard curves of glucose measured using circular dichroism at various wavelengths (200-197 nm)

Appendix 3D: Spectrum for 50 mM glucose and the reaction mix

Appendix 1A: Data for BSA binding using MilliQ water and TEA pH 8.0

Table 1A.1: MilliQ water binding

Batch 1					mg. ml ⁻¹				mg. mg ⁻¹
	OD 280 nm			average	unbound	bound	ionic	covalent	
BSA	0.495	0.463	0.485	0.481					
Prep A	0.047	0.047	0.048	0.047	1.956	18.044	6.034	12.011	2.681
Prep B	0.039	0.042	0.033	0.038	1.567	18.433	4.880	13.552	1.726
Prep C	0.254	0.267	0.272	0.264	10.985	9.015	2.377	6.638	0.988
Prep D	0.311	0.317	0.312	0.313	13.037	6.963	4.593	2.368	0.320
Batch 2									
	OD 280 nm			average	unbound	bound	ionic	covalent	mg. mg ⁻¹
BSA	0.462	0.472	0.479	0.471					
Prep A	0.026	0.030	0.022	0.026	1.117	18.883	6.817	12.066	2.540
Prep B	0.016	0.015	0.019	0.017	0.721	19.279	4.158	15.121	1.691
Prep C	0.281	0.294	0.299	0.291	12.362	7.638	1.907	5.731	0.952
Prep D	0.309	0.308	0.312	0.310	13.14	6.860	4.726	2.134	0.288
BATCH 3									
	OD 280 nm			average	unbound	bound	ionic	covalent	mg. mg ⁻¹
BSA	0.428	0.447	0.421	0.432					
Prep A	0.157	0.144	0.136	0.146	6.754	13.246	1.414	11.832	2.647
Prep B	0.080	0.086	0.075	0.081	3.732	16.268	1.025	15.244	1.717
Prep C	0.318	0.280	0.276	0.292	13.493	6.507	0.756	5.752	0.910
Prep D	0.385	0.387	0.388	0.387	17.887	2.113	0.116	1.997	0.270

Table 1A.2: TEA BSA binding

Batch 1					mg. ml ⁻¹				mg. mg ⁻¹
Sample	OD 280 nm			average	unbound	bound	ionic	covalent	
BSA	0.41	0.412	0.404	0.409					
Prep A	0.104	0.102	0.098	0.101	4.959	15.041	6.816	8.225	1.800
Prep B	0.122	0.128	0.126	0.125	6.134	13.866	6.878	6.988	0.816
Prep C	0.256	0.262	0.264	0.261	12.757	7.243	3.566	3.677	0.579
Prep D	0.374	0.368	0.376	0.373	18.238	1.762	0.796	0.966	0.130
Batch 2									
Sample	OD 280 nm			average	unbound	bound	ionic	covalent	mg. mg ⁻¹
BSA	0.396	0.414	0.426	0.412					
Prep A	0.098	0.104	0.11	0.104	5.049	14.951	7.510	7.441	1.628
Prep B	0.14	0.158	0.176	0.158	7.670	12.330	4.188	8.142	0.951
Prep C	0.278	0.284	0.284	0.282	13.689	6.311	1.914	4.397	0.692
Prep D	0.398	0.354	0.398	0.383	18.608	1.392	0.216	1.176	0.159
Batch 3									
Sample	OD 280 nm			average	unbound	bound	ionic	covalent	mg. mg ⁻¹
BSA	0.334	0.33	0.322	0.329					
Prep A	0.096	0.086	0.092	0.091	5.558	14.442	5.472	8.970	1.963
Prep B	0.096	0.082	0.072	0.083	5.071	14.929	6.226	8.703	1.017
Prep C	0.288	0.25	0.237	0.258	15.724	4.276	1.650	2.626	0.414
Prep D	0.308	0.316	0.308	0.311	18.905	1.095	0.018	1.077	0.145

*The calculations were based on the absorbance at 280 nm using the direct standard ratio protein content, were the absorbance of the remaining protein in the supernatant was divided by the original solution of the standard multiplied by the total concentration of the loaded protein

Appendix 2A: Standard curve of GDH in MilliQ water pH 8.0

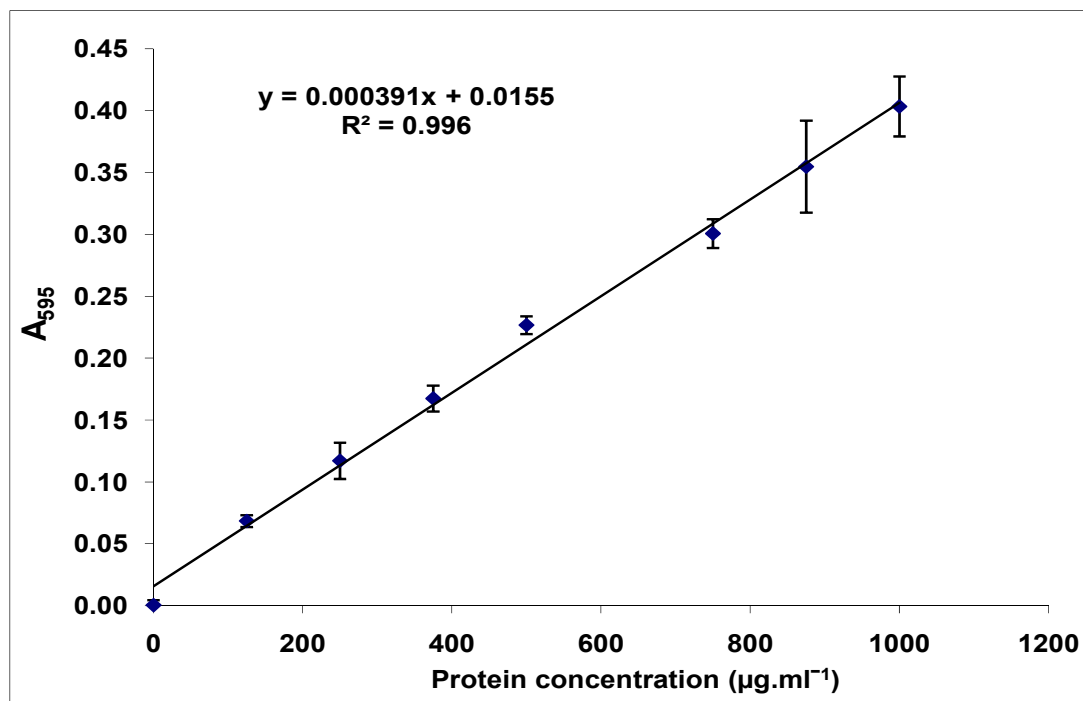


Figure 1B.1: Standard curve of GDH in MilliQ water pH 8.0

Table 1B.1: GDH binding in MilliQ water

Preparation					$\mu\text{g. ml}^{-1}$				mg. mg^{-1}
	A_{595}			Average	Unbound	Bound	Ionic	Covalent	
A	0.043	0.040	0.045	0.043	103.56	896.44	338.19	558.25	1.22
B	0.052	0.055	0.051	0.053	127.83	872.17	436.89	435.28	0.51
C	0.047	0.047	0.051	0.048	117.31	882.69	258.09	624.60	0.82
D	0.175	0.135	0.194	0.168	407.77	592.23	13.75	578.48	0.26

Appendix 2B: Standard curve of GDH in TEA buffer pH 8.0

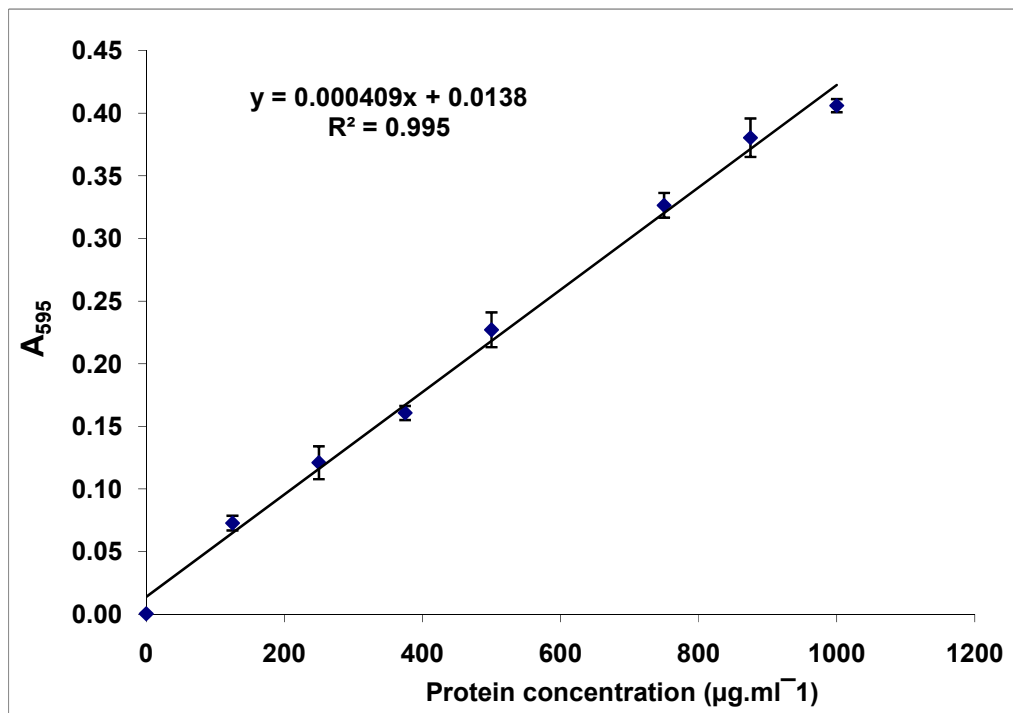


Figure 1B.1: Standard curve of GDH in TEA buffer pH 8.0

Table 1B.1: GDH binding in TEA

Preparation					$\mu\text{g. ml}^{-1}$				mg. mg^{-1}
	A_{595}			Average	Unbound	Bound	Ionic	Covalent	
A	0.058	0.054	0.052	0.055	127.73	872.27	352.80	519.47	1.137
B	0.165	0.16	0.161	0.162	378.50	621.50	318.54	302.96	0.354
C	0.221	0.224	0.225	0.223	521.81	478.19	166.67	311.53	0.409
D	0.351	0.358	0.311	0.340	794.39	205.61	0.00	205.61	0.092

Appendix 2C: Standard curve of BSA in MilliQ water pH 8.0

Used for calculating the immobilisation capacity of NOD

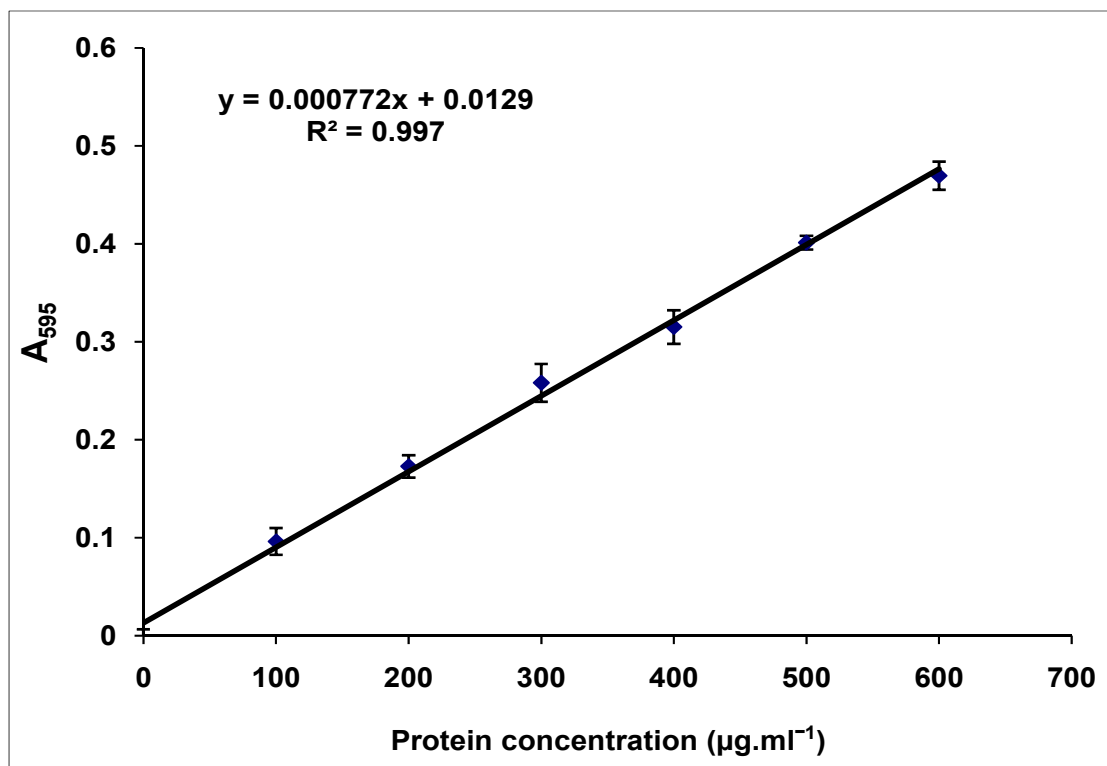


Figure 1B.1: Standard curve of BSA in MilliQ water pH 8.0

Table 1B.1: NADH Oxidase binding

Preparation					µg. ml ⁻¹				mg. mg ⁻¹
	A ₅₉₅			Average	unbound	bound	ionic	covalent	
A	-0.01	-0.003	-0.003	-0.005	-6.650	120.000	40.732	79.268	1.74
B	0.033	0.009	0.004	0.015	19.119	126.881	16.625	110.256	1.29
C	0.011	0.015	0.017	0.014	17.872	138.128	0.000	138.128	0.84

Appendix 3A: Determination of enzyme loading and amount of Prep B particles required for co-factor recycling construction

GDH		NOD
33.22	Specific activity (U.mg ⁻¹ protein)	6.75
0.51	Binding capacity (mg.mg ⁻¹)	1.29
0.203 (ratio of specific activity)	→	1
X	→	0.215 mg.ml ⁻¹ (NOD)
X	=	0.0436 mg.ml ⁻¹ (GDH)
0.0436 mg.ml ⁻¹		0.215mg.ml ⁻¹
↓		↓
0.51 mg.mg ⁻¹		1.29 mg.mg ⁻¹
↓		↓
0.0855 mg	particles required	0.167 mg
	+	
0.253 mg total particles required + 20% excess		

Appendix 3B: Assay design for the detection of D(+) glucose using Chirascan CD spectrophotometer

The absorption of light by a solute in a solution is described by the Beer-Lambert Law,

$$A = \log \frac{T(\text{ref})}{T(\text{sample})} = \epsilon c l$$

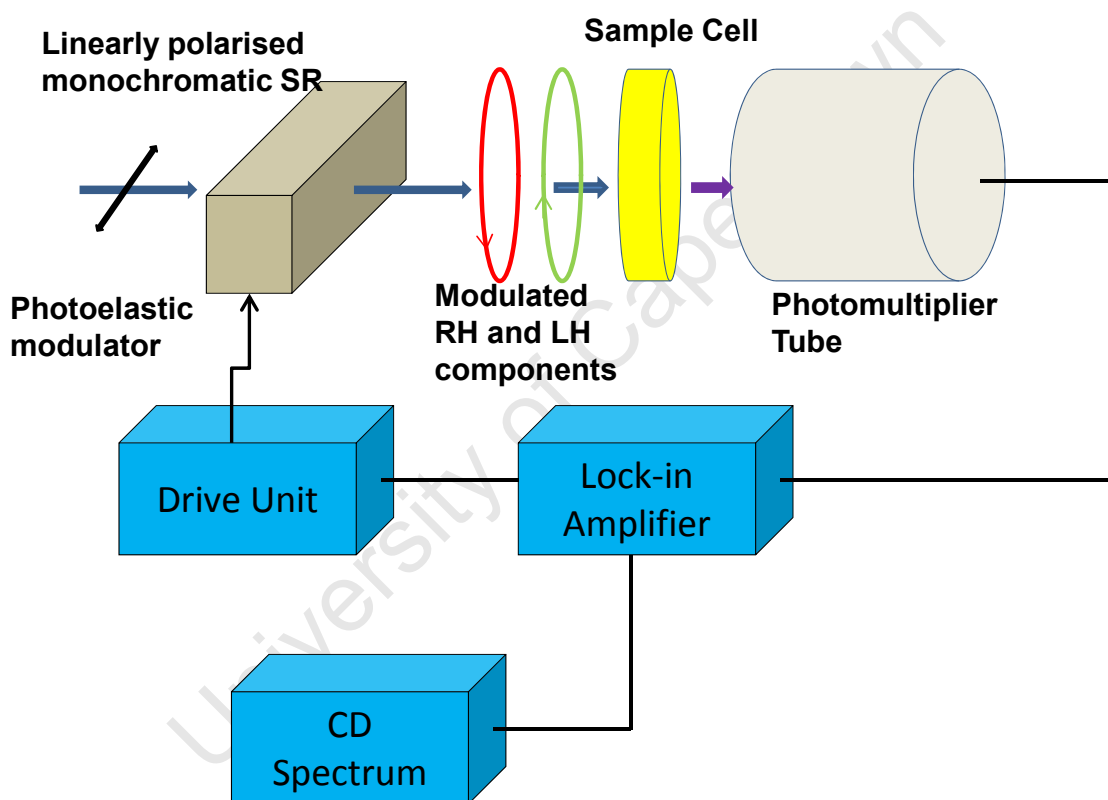
A = absorbance of the solute, T(ref) = light transmission with no sample only solvent. T(sample) = transmission with sample involved. ϵ = molar extinction coefficient of the solute at a certain wavelength, c is the molar concentration and l is the length of the light path in cm. In chiral molecules the value of the molar extinction coefficient is different for the left and right polarised light, ϵ_L and ϵ_R . The difference in this value, $\Delta\epsilon = \epsilon_L - \epsilon_R$, represents a CD spectrum (and for non chiral molecules $\epsilon_L = \epsilon_R$ hence no CD spectrum). spectropolarimeter or CD spectrophotometer (Voet & Voet, 2004).

The measurement of absorbance during CD measurement provides an indication of the transmission of the sample and any solvents present over the wavelength being investigated on the Chirascan (Applied Photophysics, UK, Schematic 3B.1). The absorbance measurement aids in validating the CD measurement, since a lack of light throughput will decrease the AC and DC signals to meaningless levels resulting in incorrect CD values. It has been noted that the optimum CD measurement are obtained where the absorbance of the sample is about 0.8 – 1.0 (a.u). If the absorbance is too low the CD signal will be weak and if it too high it will limit the light reaching the detector (N.B.: Chirascan has an absorbance limit of 5.5 (a.u)).

The various parts of the machine have different functions. The monochromator yields a pure linear polarised monochromatic light and allows high spectral bandwidths in the far-UV (260 – 175 nm). This generated light is re-focussed down through the photoelastic modulator which modulates the beam into alternately left and right

circularly polarised states. This beam passes into the sample housing, passing through the sample holder and then onto the photomultiplier CD detector.

The light beam is converted into a photocurrent which is proportional to the incident light flux. If there is a differential transmission of the two circularly modulated states, left and right, this results in the AC component superimposed on a background steady state DC component. The CD is calculated from the ratio of the AC and DC components (Chirascan manual updated July 2004).



Schematic 3B.1: Schematic diagram of the CD spectrophotometer.

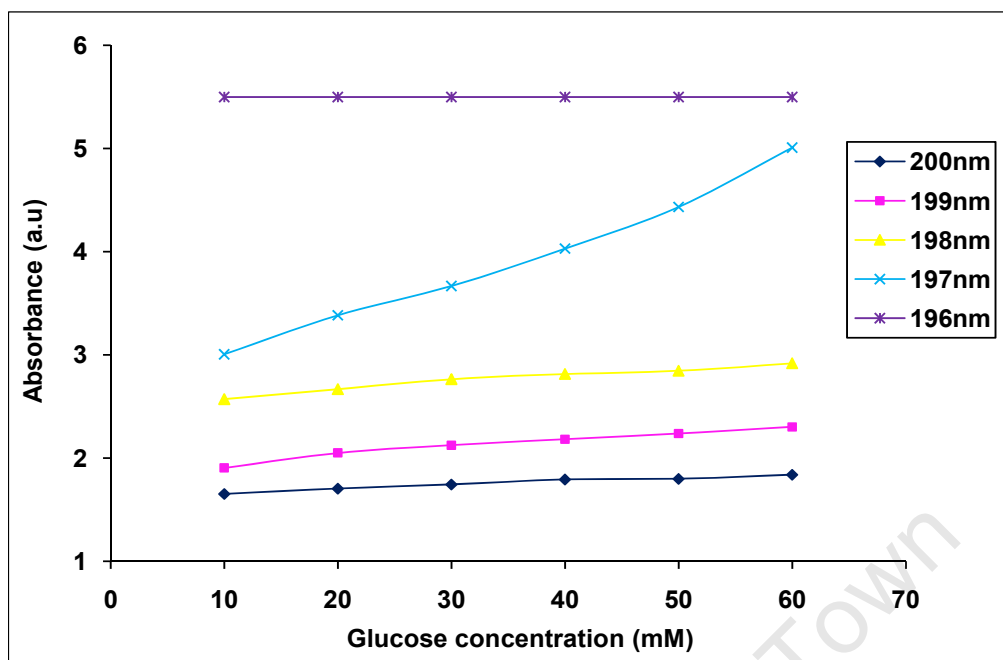


Figure 3B.2: Absorbance of varied concentrations of D(+)-glucose at different wavelengths. The absorbance of the glucose at wavelengths 195-193 nm was similar to that off 196 nm.

Appendix 3C: Standard curves of glucose measured using circular dichroism at various wavelengths (200-193 nm)

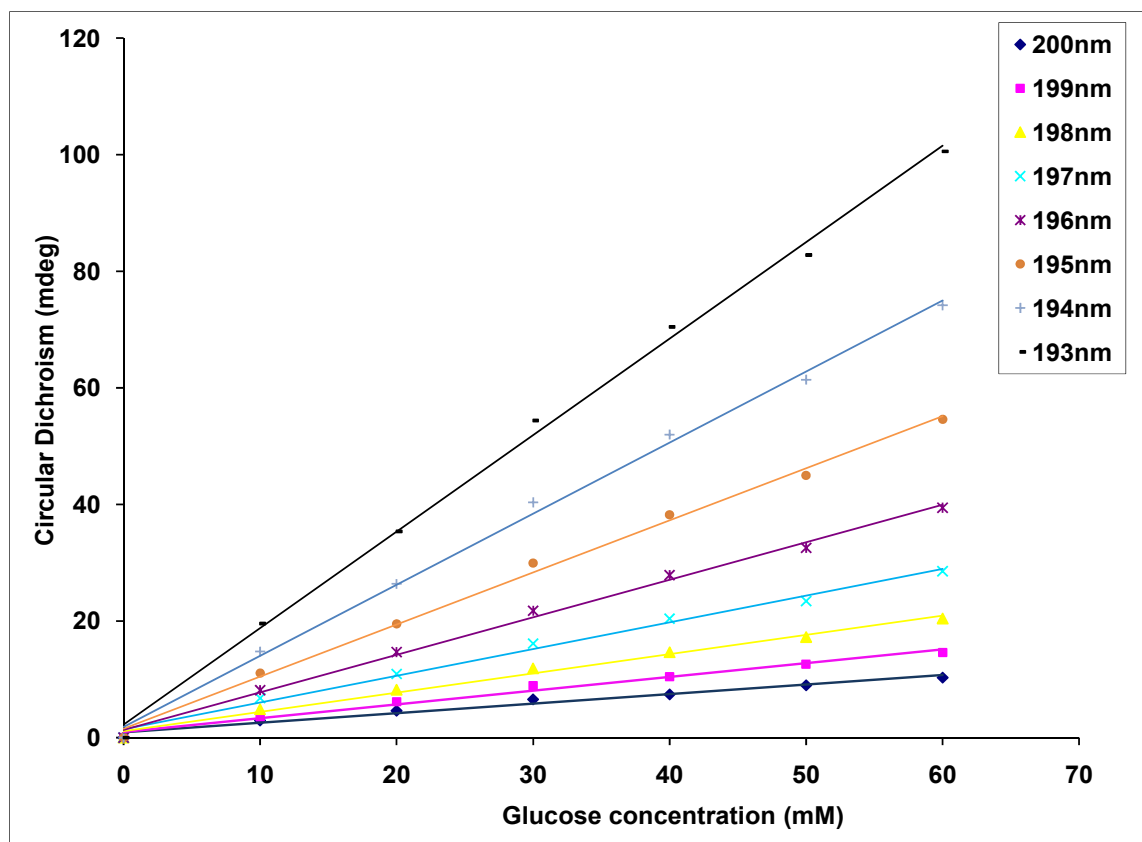


Figure 3C.1: Standard curves of glucose using CD at various wavelengths (200-193).

Wavelength (nm)	Equation	R ²
200	$y = 0.163x + 0.929$	0.975
199	$y = 0.236x + 0.948$	0.986
198	$y = 0.330x + 1.139$	0.990
197	$y = 0.458x + 1.412$	0.991
196	$y = 0.644x + 1.310$	0.995
195	$y = 0.894x + 1.522$	0.996
194	$y = 1.219x + 1.860$	0.997
193	$y = 1.655x + 2.242$	0.997

Appendix 3D: Spectrum for 50 mM glucose and the reaction mix

There was interference from 193-196 nm which was likely due to the high absorbance of the reaction mix, resulting in no CD measurement. No interfering compounds from 197 nm.

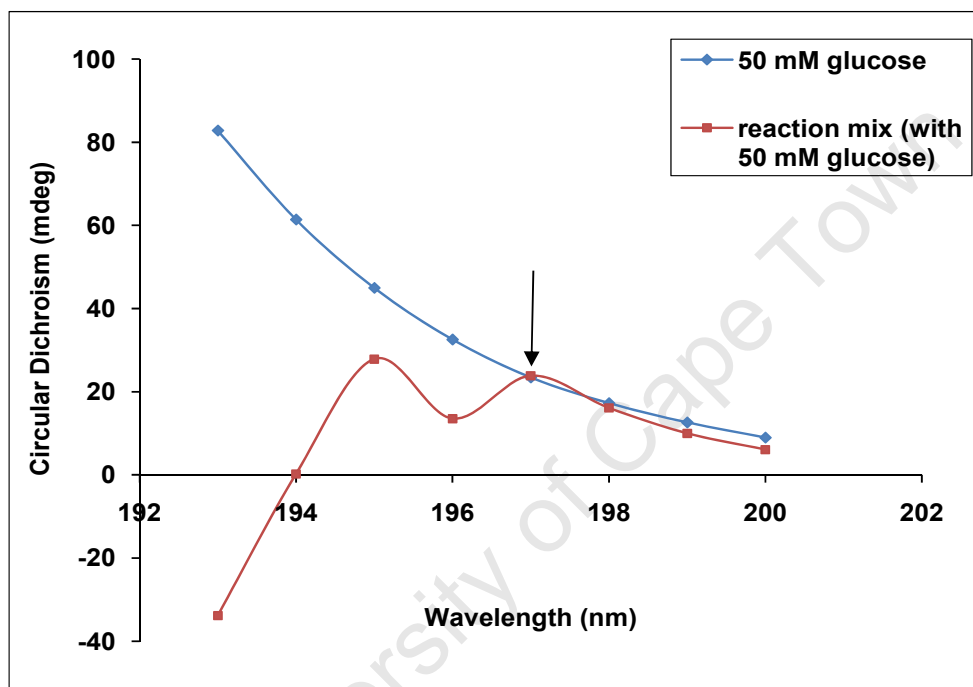


Figure 3D.1: CD spectrum of the starting concentration of glucose used in the assay with and without all the reaction components. The CD values of the two solutions corresponded mainly at 197 nm.

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